EFFECTS OF PANTOTHENIC ACID DEFICIENCY ON PITUITARY-ADRENAL FUNCTION IN THE RAT[1]

B. H. ERSHOFF, R. B. ALFIN SLATER AND J. G. GAINES

Emory W. Thurston Laboratories, Los Angeles, and the Department of Biochemistry and Nutrition, University of Southern California, Los Angeles

(Received for publication January 22, 1953)

Considerable data are available indicating that adrenocortical function is impaired in the pantothenic acid-deficient rat. The subject has recently been reviewed and pertinent references cited (Morgan, '51; Cowgill et al., '52; Ershoff, '52). In the present communication further data are presented on the effects of pantothenic acid deficiency on adrenocortical function in the rat. Findings indicate that although various indices of pituitary-adrenal activation are abnormal in the pantothenic acid-deficient rat, such animals may nevertheless produce substantial amounts of a cortisone-like hormone or hormones.

PROCEDURE AND RESULTS

Experiment 1. Comparative effects of epinephrine and ACTH administration on the peripheral blood count of pantothenic acid-deficient rats

It is well established that in the normal animal activation of the pituitary-adrenal system results in a reduction of the

[1] This paper reports research undertaken in cooperation with the Office of Naval Research and the Quartermaster Food and Container Institute for the Armed Forces. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army or the Office of Naval Research. Communication 330 from the Department of Biochemistry and Nutrition, University of Southern California.

Copyright 1953
The Wistar Institute of Anatomy and Biology
All rights reserved

peripheral blood lymphocyte count. This reduction is apparently due to an increased production of adrenal corticosteroids, inasmuch as corticosterone and ACTH administration both duplicate this effect (Dougherty and White, '44; Reinhardt et al., '44; Palmer et al., '51). Administration of epinephrine or exposure to any one of a number of non-specific stressor agents also results in lymphopenia, presumably due at least in part to ACTH discharge (Selye, '50, '51). Alterations in the peripheral lymphocyte count after epinephrine, ACTH or cortisone administration have been employed as an index of pituitary-adrenal function (Winters et al., '52a; Ershoff and Parrott, '53). The rationale for such a test, employing alterations in the peripheral eosinophile count as an index, had previously been established (Recant et al., '50). If lymphopenia fails to occur following the injection of epinephrine but does result after ACTH administration, it might indicate impaired response at the pituitary level (inadequate ACTH secretion). If lymphopenia occurs after cortisone administration but not after the injection of ACTH, it might indicate failure of response at the adrenal level (impaired formation of a cortical hormone or hormones). The absence of lymphopenia after cortisone administration might indicate failure of response at the peripheral tissue level.

The above test has been applied to pantothenic acid-deficient rats, with conflicting results. Winters et al. ('52a) observed little if any reduction in the peripheral lymphocyte (and eosinophile) count of pantothenic acid-deficient rats after either epinephrine or ACTH administration, in contrast to the marked change in animals receiving cortisone. A decreased lymphocyte response in pantothenic acid-deficient rats after ACTH administration had previously been reported by Dumm et al. ('49). Ershoff and Parrott ('53), however, found no impairment in the lymphocyte response of pantothenic acid-deficient rats injected with ACTH, but a marked impairment after epinephrine administration. The following experiment was accordingly undertaken to obtain

further data on the comparative effects of epinephrine and ACTH administration on the peripheral lymphocyte count of the pantothenic acid-deficient rat.

The basal ration employed in the present experiment consisted, in per cent, of sucrose, 61; casein,[2] 24; salt mixture,[3] 5; cottonseed oil,[4] 8; and wheat germ oil,[5] 2. To each kilogram of the above diet were added the following synthetic vitamins: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; nicotinic acid, 60 mg; biotin, 5 mg; 2-methyl-naphthoquinone, 5 mg; folic acid, 10 mg; ascorbic acid, 100 mg; *p*-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin B_{12}, 100 μg; and choline chloride, 2 gm. To each kilogram of diet were also added 6,000 U.S.P. units of vitamin A[6] and 600 U.S.P. units of vitamin D.[7] The vitamins were added in place of an equal amount of sucrose. Tests were conducted: (1) with rats fed the pantothenic acid-free basal ration indicated above, and (2) with animals fed a similar diet supplemented with calcium pantothenate at a level of 60 mg per kilogram of ration.

Eighty-six female rats of the Long-Evans strain were selected at 21 to 23 days of age and at a body weight of 38 to 50 gm for the present experiment. The animals were divided into three groups. Group A (consisting of 46 rats) was fed ad libitum the basal pantothenate-free ration indicated above; group B (consisting of 20 rats) was fed ad libitum the complete ration (basal ration + calcium pantothenate); and group C (consisting of 20 rats) was pair-fed the complete ration in amounts equal to that ingested daily by rats on the calcium pantothenate-free diet. Animals were kept in individual metal cages with raised screen bottoms

[2] Vitamin-free test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Hubbel, Mendel and Wakeman salt mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Wesson.

[5] VioBin.

[6] MYVA-DRY Powder, Distillation Products, Rochester, New York.

[7] HY-DEE Powder, Standard Brands, New York, N. Y.

to prevent access to feces. Diets were made up weekly and stored under refrigeration when not in use. Rats were fed daily. All food not consumed 24 hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet.

After 30 days of feeding the average body weights of rats in the three groups were as follows: group A, 82.6 gm (range, 62 to 112 gm); group B, 149.6 gm (range, 128 to 165 gm); and group C, 112.3 gm (range, 106 to 122 gm). The average daily food consumption per rat during this period was 8.7 gm per day for animals in groups A and C and 12.5 gm per day for animals in group B. Four rats in group A had succumbed. Of the remaining 42 animals, 34 had been depleted of pantothenic acid as judged by stationary or decreasing body weight for a period of 5 days.[8] All rats in groups B and C had survived (animals in group C, however, had reached a plateau in body weight).

At this time the 34 depleted rats in group A and all rats in groups B and C were selected for the following experiment. Half the animals in each group received a single intraperitoneal injection of 0.03 mg epinephrine per 100 gm body weight[9]; the remaining half received a single intraperitoneal injection of ACTH[10] equivalent to 4 mg of Armour's standard per 100 gm body weight. Immediately before the injection and at two, 4 and 24 hours afterward, specimens were taken of the tail blood of all rats, and total and differential white cell counts determined. Differential counts were made on smears stained with Wright's stain, 100 cells on each of two slides being employed for each analysis. The blood specimens were taken from animals after subjecting them to total

[8] Henceforth in this paper the term "pantothenic acid-depleted" is applied to rats that had reached a plateau in body weight as judged by stationary or decreasing body weight for a period of 5 days while on pantothenic acid-deficient diet.

[9] Adrenalin chloride solution, 1: 1000 adrenalin, Parke, Davis and Co., Detroit, Michigan.

[10] Acthar, Armour and Co., Chicago, Illinois.

body heating as previously described (Ershoff and Gaines, '53).[11]

Results are summarized in table 1. Findings indicate that under the conditions of the present experiment pantothenic acid deficiency did not impair the lymphopenic response to ACTH administration in the rat. The reduction in the lymphocyte count of rats depleted of pantothenic acid did not differ significantly from that of rats fed the complete diet ad libitum and was greater than that in rats pair-fed the complete diet. If a reduction in the lymphocyte count is accepted as an index of adrenocortical activation, it would appear that under the conditions of the present experiment pantothenic acid deficiency did not impair the ability of the adrenal cortex to respond to corticotrophin stimulation (at least insofar as the production of lymphopenia-producing corticoids is concerned). These findings confirm those previously reported from this laboratory when localized tail heating was employed in obtaining the blood specimen (Ershoff and Parrott, '53) but differ from the observations of Winters et al. ('52a). It is not readily apparent to what extent such factors as differences in the diet, degree of pantothenic acid deficiency, age and strain of the experimental animal or dosage of ACTH employed were responsible for the diverse results.

In the epinephrine series a clear-cut distinction was observed between the lymphocyte response of pantothenic acid-deficient rats and that of animals fed the complete ration either ad libitum or at a restricted caloric intake. On the latter ration a significant reduction in lymphocytes was noted two hours post-injection in both the ad libitum and pair-fed groups. Rats depleted of pantothenic acid, however, exhibited no lymphopenia two hours post-injection but, on the contrary, a marked lymphocytosis. Inasmuch as pantothenic

[11] This method of obtaining the blood specimen has the advantage of providing a profuse flow of blood on severing the tip of the tail. It is of particular value when blood specimens are to be taken from animals in a poor nutritive state. White blood cell counts obtained by this technique, however, are considerably smaller than those generally reported for peripheral blood in the rat and more comparable to the values obtained for heart blood (Ershoff and Gaines, '53).

TABLE 1

Comparative effects of epinephrine and ACTH on the peripheral blood count of pantothenic acid-deficient rats[1]

DIETARY GROUP	NUMBER OF ANIMALS	BODY WT. AT TIME OF INJECTION	AVERAGE TOTAL LEUKOCYTES	AVERAGE GRANULOCYTES		AVERAGE LYMPHOCYTES	AVERAGE CHANGE IN LYMPHOCYTE COUNT FROM PRE-INJECTION LEVEL
			Epinephrine series				
		gm		%	*total*		%
Pantothenic acid-deficient	14	74					
Pre-injection			4,425	27	1,195	3,230	..
2 hours post-injection			10,407	62	6,452	3,955	+ 22
4 hours post-injection			10,388	74	7,687	2,701	— 16
24 hours post-injection			3,736	36	1,345	2,391	— 26
Complete ration ad-libitum	10	162					
Pre-injection			5,563	26	1,446	4,117	..
2 hours post-injection			11,893	73	8,682	3,211	— 22
4 hours post-injection			10,593	67	7,097	3,496	— 15
24 hours post-injection			5,600	30	1,680	3,920	— 5
Complete ration pair-fed	10	116					
Pre-injection			4,171	26	1,084	3,087	
2 hours post-injection			12,107	78	9,443	2,664	— 14
4 hours post-injection			10,593	69	7,309	3,284	+ 6
24 hours post-injection			4,293	27	1,159	3,134	+ 2
			ACTH series				
Pantothenic acid-deficient	13	71					
Pre-injection			4,868	30	1,460	3,408	..
2 hours post-injection			6,238	57	3,556	2,682	— 21
4 hours post-injection			5,258	59	3,102	2,156	— 37
24 hours post-injection			4,450	40	1,780	2,670	— 22
Complete ration ad-libitum	10	166					
Pre-injection			6,208	25	1,552	4,656	..
2 hours post-injection			10,090	71	7,164	2,926	— 37
4 hours post-injection			9,542	72	6,870	2,672	— 43
24 hours post-injection			5,950	38	2,261	3,689	— 21
Complete ration pair-fed	10	118					
Pre-injection			4,628	20	926	3,702	
2 hours post-injection			5,943	50	2,972	2,971	— 20
4 hours post-injection			6,825	56	3,822	3,003	— 19
24 hours post-injection			5,421	27	1,464	3,957	+ 7

[1] Data for total leukocytes, lymphocytes and total granulocytes are expressed as cells/mm³.

acid-deficient rats showed a significant reduction in blood lymphocytes two hours after the administration of ACTH, it would appear that the failure of pantothenic acid-deficient rats to exhibit lymphopenia two hours after epinephrine administration was due, not to an impaired ability on the part of the adrenal cortex of these animals to respond to cortical stimulation by the production of lymphopenia-producing corticoids, but rather to an inadequate ACTH secretion. Four hours after epinephrine administration, however, the lymphocyte count of pantothenic acid-deficient rats was significantly reduced from pre-injection levels; and by the 24th hour post-injection, the average reduction in lymphocyte count for these animals was equal to the maximum reduction observed on the complete ration (which occurred two hours after epinephrine injection). These findings suggest that pantothenic acid deficiency does not prevent the synthesis or secretion of ACTH in the rat, but that it does modify the rate at which ACTH is discharged or secreted following epinephrine administration. It is apparent that conclusions based on the lymphocyte count two hours after epinephrine administration in the pantothenic acid-depleted rat would certainly differ from those obtained on the basis of the maximum reduction following epinephrine injection.

Experiment 2. Comparative effects of epinephrine and ACTH administration on adrenal ascorbic acid and cholesterol of pantothenic acid-deficient rats

It is well established that the administration of ACTH is followed by a reduction in the ascorbic acid and cholesterol content of the adrenals proportional, in general, to the amount of material administered (Long, '47; Sayers and Sayers, '48). A similar reduction occurs after the administration of epinephrine or exposure to hemorrhage, burns and other forms of stress, due presumably to ACTH discharge, since such effects do not occur in hypophysectomized animals

[1] Data for total leukocytes, lymphocytes and total granulocytes are expressed as cells/mm³.

subjected to the above stressor agents, whereas ascorbic acid and cholesterol are depleted from the adrenals of hypophysectomized animals injected with ACTH. The reduction in adrenal ascorbic acid and cholesterol indicated above is associated with evidence of increased cortical hormone secretion (as judged by lymphopenia and increase in the liver glycogen of fasted animals 6 to 9 hours after ACTH administration or induction of stress). Although there is evidence that adrenals depleted of ascorbic acid can still respond to ACTH administration with cortical hormone secretion (as judged by the development of lymphopenia), ascorbic acid depletion is nevertheless regarded as one of the best indices of adrenocortical function currently available. The following experiment was undertaken to determine the effects of pantothenic acid deficiency on the ascorbic acid and cholesterol content of the adrenals following epinephrine and ACTH administration in the rat.

One hundred and sixty female rats of the Wistar strain were selected at 21 to 23 days of age and at a body weight of 38 to 50 gm for the present experiment. Female rats were selected since they had larger adrenals than the males. Animals of the Wistar strain were employed on the basis of preliminary findings that the adrenal glands are more uniform in respect to weight and cholesterol content in this strain than in animals of the Long-Evans strain. The experimental diets and the procedure employed were similar to those described in experiment 1. Group A (consisting of 60 rats) was fed ad libitum the basal pantothenate-free ration; group B (consisting of 50 rats) was fed ad libitum the complete ration (basal ration + calcium pantothenate); and group C (consisting of 50 rats) was pair-fed the complete ration in amounts equal to that ingested daily by rats on the pantothenate-free diet. By the 24th day of feeding 52 of the 60 rats in group A had been depleted of pantothenic acid.[12]

[12] Of the remaining 8 animals, three had succumbed, while 5 were still gaining weight.

At this time 50 of the depleted rats in group A and all the rats in groups B and C were divided into 5 subgroups of 10 rats each and treated as follows. Subgroup 1 served as pre-injection controls; subgroups 2 and 3 received a single intraperitoneal injection of epinephrine; and subgroups 4 and 5 received a single intraperitoneal injection of ACTH. Subgroups 2 and 4 were autopsied one hour post-injection; subgroups 3 and 5 were autopsied three hours post-injection. Epinephrine was administered at a level of 0.03 mg per 10 gm body weight; ACTH at a level of 4 mg (Armour's standard) per 100 gm body weight.

Animals were anesthetized with nembutal and sacrificed by withdrawal of blood by heart puncture. The adrenal glands were quickly extirpated and adhering fat was removed. Each adrenal was weighed to the nearest 0.2 mg on a torsion balance; the left adrenal thereafter was used for analysis of ascorbic acid content, and the right adrenal was used for total cholesterol analysis. Ascorbic acid determinations were done by the Roe and Kuether method ('43) on a 4% trichloroacetic acid extract of the adrenal gland, and cholesterol analyses were performed on the alcohol-ether extract of the gland by a modified Sperry-Schoenheimer method as described by Nieft and Deuel ('50). Hemorrhagic adrenals were observed grossly in approximately one-fifth of the pantothenic acid-depleted rats. Cholesterol and ascorbic acid values obtained on these adrenals were not included in the data presented in table 2.

Findings indicate (table 2) that adrenal ascorbic acid was reduced one and three hours post-injection in all groups after epinephrine and ACTH administration. In the ACTH series, adrenal ascorbic acid was reduced by approximately 40% from pre-injection levels in both groups fed the complete ration (groups B and C); pantothenic acid-depleted rats (group A), however, exhibited a reduction of only 23 and 26% after one and three hours, respectively, under similar conditions. These findings suggest that the response of the adrenal cortex to corticotropin stimulation was impaired in

TABLE 2

Effects of pantothenic acid deficiency on the ascorbic acid and cholesterol content of the adrenals following ACTH and epinephrine administration in the rat

EXPERIMENTAL GROUP	BODY WT. AT TIME OF INJECTION		ADRENAL ASCORBIC ACID		CHANGE IN ADRENAL ASCORBIC ACID FROM PRE-INJECTION LEVEL	ADRENAL CHOLESTEROL		CHANGE IN ADRENAL CHOLESTEROL FROM PRE-INJECTION LEVEL
	Ave.	Range	Ave.	Range		Ave.	Range	
	gm		*μg/100 mg*		%	*mg cholesterol per gm adrenal wt.*		%
Pantothenic acid-deficient	84.2 [1] (50)	61–102						
Pre-injection			427 (9)	375–447	..	13.7 (9)	7.3–18.0	..
ACTH-1 hour post injection			328 (8)	224–388	— 23	14.7 (9)	8.1–21.3	+ 7
ACTH-3 hours post injection			317 (7)	231–360	— 26	17.6 (7)	7.5–25.0	+ 28
Epinephrine-1 hour post injection			301 (9)	208–392	— 30	18.4 (9)	9.6–32.3	+ 34
Epinephrine-3 hours post injection			229 (8)	154–375	— 46	17.2 (8)	7.6–26.0	+ 26
Complete ration — ad libitum	168.9 (50)	151–183						
Pre-injection			392 (8)	387–400	..	38.9 (10)	33.1–46.4	..
ACTH-1 hour post injection			227 (9)	194–281	— 42	22.1 (10)	14.4–27.0	— 43
ACTH-3 hours post injection			219 (10)	201–235	— 44	27.7 (10)	22.0–32.0	— 29
Epinephrine-1 hour post injection			250 (9)	230–266	— 36	33.6 (10)	25.7–42.4	— 14
Epinephrine-3 hours post injection			236 (9)	222–264	— 40	25.1 (10)	13.2–32.0	— 35
Complete ration — pair-fed	97.5 (50)	89–108						
Pre-injection			433 (9)	347–510	..	26.2 (8)	20.6–30.9	..
ACTH-1 hour post injection			249 (9)	185–387	— 42	30.7 (10)	16.8–47.0	+ 17
ACTH-3 hours post injection			259 (10)	216–371	— 40	28.3 (10)	15.4–36.3	+ 8
Epinephrine-1 hour post injection			322 (9)	265–366	— 26	22.9 (8)	16.3–29.1	— 13
Epinephrine-3 hours post injection			317 (10)	291–436	— 27	24.3 (10)	16.8–26.5	— 7

[1] The numbers in parentheses indicate the number of animals on which values are based.

the pantothenic acid-depleted rat. Data obtained after epinephrine administration, however, do not support this hypothesis. In this series the reduction in adrenal ascorbic acid from pre-injection levels was as marked in pantothenic acid-depleted rats (group A) as that observed in rats fed the complete ration ad libitum (group B) and greater than that observed in pair-fed controls (group C). The cause for these divergent results is not readily apparent. Inasmuch as the reduction in adrenal ascorbic acid is believed to be a specific response to corticotropin stimulation, it would appear that no impairment existed in pantothenic acid-depleted rats either in the amount of ACTH discharged after epinephrine administration or in the response of the adrenals thereto.

The adrenal cholesterol of uninjected rats deficient in pantothenic acid was significantly less than that of rats fed a complete ration ad libitum or a similar ration restricted in amount to that ingested daily by rats on the pantothenic acid-deficient diet. The average values for the three groups were as follows: pantothenic acid-deficient (group A), 13.7 mg per gram adrenal weight; complete ration ad libitum (group B), 38.9 mg per gram adrenal weight; and complete ration pair-fed (group C), 26.2 mg per gram adrenal weight. In the complete ration ad libitum series (group B), a significant depression of adrenal cholesterol occurred following epinephrine and ACTH administration both in the one-hour and three-hour post-injection groups. Rats depleted of pantothenic acid (group A), however, showed no reduction in adrenal cholesterol values following epinephrine or ACTH administration. These findings suggest that pituitary-adrenal function was impaired in the pantothenic acid-depleted rat and that the impairment was due, at least in part, to a defective response on the part of the adrenal cortex to corticotropin stimulation.

There are reasons, however, to doubt the validity of this hypothesis. Inasmuch as the cholesterol content of the adrenals of uninjected pantothenic acid-depleted rats is on the average only one-third that of normal controls, it is ques-

[1] The numbers in parentheses indicate the number of animals on which values are based.

tionable whether the failure of such adrenals to exhibit a further reduction following epinephrine or ACTH administration is indicative of impaired adrenocortical activation. Furthermore, rats fed a restricted intake of the complete ration (group C) also failed to show a reduction in adrenal cholesterol concentration following ACTH administration, although a decrease occurred following the administration of epinephrine. It would appear from the latter findings that the failure of pantothenic acid-depleted rats to exhibit a reduction in adrenal cholesterol concentration following ACTH administration was due not to pantothenic acid deficiency per se but, at least in part, under the conditions of the present experiment, to the accompanying reduction in caloric intake. It is not readily apparent why rats in group C exhibited a reduction in adrenal cholesterol values after epinephrine administration but not after ACTH.

Experiment 3. Comparative effects of egg white toxicity in pantothenic acid-deficient and adrenalectomized rats

It is well established that adrenalectomized rats rapidly succumb following the parenteral administration of egg white (Selye, '37, '49) but that such animals will survive if cortisone is also administered (Selye, '49). The protection afforded adrenalectomized rats from lethal doses of egg white appears to be specific for cortisone and its related compounds and has been employed as a functional test for cortisone activity in the rat (Chen and Wickel, '52). The following experiment was conducted to determine the comparative effects of adrenalectomy and pantothenic acid deficiency on resistance to egg white intoxication in the rat. It was felt that if the synthesis of cortisone-like hormones were impaired in the pantothenic acid-depleted rat, such animals would resemble the adrenalectomized rat in their response and would rapidly succumb following egg white administration.

a. Effects of adrenalectomy on resistance to egg white intoxication. Twenty-one female rats of the Long-Evans strain which had been raised from weaning on a natural food ration were selected at 80 to 90 gm in weight for the present experiment. Fourteen of these animals were bilaterally adrenalectomized; the remaining 7 served as intact controls. All adrenalectomized rats received 1% NaCl as drinking fluid during the course of the experiment. Seventy-two hours after the operation fresh egg white (2 ml of a one-to-one dilution per 100 gm body weight) was injected intraperitoneally in all rats. Seven of the adrenalectomized rats, however, had received 5 mg of cortisone acetate [13] administered intraperitoneally two hours prior to the egg white injection. The number of deaths in each group was then recorded for the ensuing 96 hours. Of the 7 adrenalectomized rats that did not receive cortisone, 5 animals died within 3 hours of the time that egg white was administered, and only one animal survived the experimental period of 4 days. In contrast, all intact animals and all adrenalectomized rats that received cortisone survived.

b. Effects of pantothenic acid deficiency on resistance to egg white intoxication. The procedure and diet employed for the following animals were similar to those described in experiment 1. Female rats of the Long-Evans strain were placed at weaning on the purified pantothenate-free ration and were selected for the present experiment at depletion. Twenty pantothenic acid-depleted rats (consisting of two groups of 10 animals each) were employed. One group received fresh egg white at a level of 2 ml of a one-to-one dilution per 100 gm body weight; the second group received twice this dose. Injections were made intraperitoneally. All animals survived an experimental period of 4 days.

It is apparent from these findings that pantothenic acid-depleted rats differ from adrenalectomized rats insofar as

[13] Saline solution of cortone acetate, Merck and Co., Rahway, New Jersey, containing 25 mg cortisone acetate per milliliter. This material was kindly provided by Dr. R. A. Peterman of Merck and Co.

their ability to survive after egg white intoxication is concerned. If survival after egg white administration is dependent on a minimum concentration of cortisone or cortisone-like substances, it would appear that no impairment existed in the ability of pantothenic acid-depleted rats to produce these compounds in the amounts required for survival under the conditions of the present experiment.

DISCUSSION

The present investigation was undertaken to determine the effects of pantothenic acid deficiency on the pituitary-adrenal system of the rat. Pituitary-adrenal activation following epinephrine and ACTH administration was measured by the following indices of adrenocortical activation: (1) reduction in the peripheral lymphocyte count; (2) adrenal ascorbic acid depletion; and (3) reduction in adrenal cholesterol concentration. Findings for pantothenic acid-depleted rats were contrasted to those observed for rats fed a complete ration, either ad libitum or in an amount equal to that ingested daily by rats on the pantothenate-free ration. In addition to the above indirect measurements of adrenocortical activation, tests were conducted on the capacity of pantothenic acid-depleted rats to survive following egg white intoxication (the latter has been employed as a functional test for the presence of cortisone-like hormones in the rat).

With the exception of the cholesterol depletion studies, all tests indicate that pituitary-adrenal function was not significantly impaired in the pantothenic acid-depleted rat. The resistance of such animals to egg white intoxication was not decreased, nor did the peripheral lymphocyte response nor the extent of adrenal ascorbic acid depletion following epinephrine or ACTH administration differ significantly in pantothenic acid-depleted rats from these responses in animals on a complete ration.[14] Certain minor differences did occur in

[14] In a report which appeared while the present manuscript was in press and in which exposure to cold was employed as a stressor agent, Perry et al. ('53) also observed no impairment in the adrenal ascorbic acid depletion response of pantothenic acid-deficient rats.

the pantothenic acid-deficient group; for example, the absence of a lymphopenic response two hours after epinephrine injection and a decreased responsiveness to ACTH as measured by the adrenal ascorbic acid depletion response. In general, however, little if any impairment in pituitary-adrenal function was noted under the conditions of the present experiment in the pantothenic acid-depleted rat. This does not rule out the possibility that an impairment in pituitary-adrenal function might have occurred in animals that were more severely depleted. It must be pointed out, however, that the degree of pantothenic acid deficiency in the present experiment was quite marked as judged by such indices as the growth slope, body weight at time of depletion, gross appearance and the fact that in each series several of the pantothenic acid-depleted rats had succumbed prior to the start of the test.

Reduction in adrenal cholesterol does not appear to be a valid indicator of adrenocortical activation in the pantothenic acid-depleted animal. No reduction in adrenal cholesterol occurred following either epinephrine or ACTH administration in pantothenic acid-depleted rats, although adrenal ascorbic acid was depleted in the same animals. Since the adrenal cholesterol content of uninjected pantothenic acid-depleted rats was on the average only one-third that of normal controls, it is questionable whether the failure to obtain a further reduction in the adrenal cholesterol of pantothenic acid-depleted rats following epinephrine or ACTH administration can rightfully be interpreted as an impaired adrenocortical response.

Of the various indices of adrenocortical activation employed in the present experiment, resistance to egg white intoxication appears to be the most valid as a test for the adequacy of pituitary-adrenal function in the pantothenic acid-depleted rat. This conclusion is based on findings which indicate that survival following egg white intoxication (under the conditions of the present experiment) constitutes a functional test for the presence of cortisone-like hormones and hence provides more direct evidence as to the adequacy of

adrenocortical function than morphological or chemical alterations in the tissues (which may or may not reflect accurately the amount and physiological potency of the pituitary and adrenal hormones produced). There is evidence that a number of corticoids are present in the adrenal gland, and these may vary both in amount and kind in the cortical secretions produced in response to different stressor agents. Evidence of adrenal activation and adequacy obtained with one index, therefore, does not rule out the possibility of an impaired production of corticoids (either in amount or kind) which might be shown by other indices. Since other investigators have repeatedly demonstrated disturbances in the carbohydrate metabolism of pantothenic acid-deficient rats which could be corrected by the administration of adrenal cortical extracts or cortisone acetate (Winters et al., '52b; Krehl et al., '52; Hurley and Morgan, '52), it would appear that some impairment or dysfunction in adrenocortical function does occur in animals deficient in this vitamin. It is possible that alterations in carbohydrate metabolism may occur in the pantothenic acid-deficient rat which can be corrected by the administration of cortisone acetate without other functions of the adrenal cortex necessarily being adversely affected.

The finding in the present experiment following epinephrine and egg white administration was essentially a response of the organism to acute stress involving the discharge of preformed ACTH. It is possible, however, as Samuels ('51) has pointed out, that a nutritionally deficient animal may have sufficient ACTH in its pituitary to meet body requirements for this factor during an acute stress reaction, but that such animals may have an impaired ability to synthesize ACTH and other hormones in the amounts required during repeated or chronic stress. Further studies are indicated to determine the effects of chronic exposure to stress on pituitary-adrenal function in the pantothenic acid-deficient rat.

SUMMARY

Tests were conducted to determine the effects of pantothenic acid deficiency on the pituitary-adrenal system of the rat. Pituitary-adrenal activation following epinephrine and ACTH administration was measured by the following indices of adrenocortical activation: (1) reduction in the peripheral lymphocyte count; (2) adrenal ascorbic acid depletion; and (3) reduction in adrenal cholesterol concentration. Findings for pantothenic acid-depleted rats were contrasted to those observed for rats fed a complete ration, either ad libitum or in an amount equal to that ingested daily by rats on the pantothenate-free ration. In addition to the above indirect measurements of adrenocortical activation, tests were conducted on the capacity of pantothenic acid-depleted rats to survive following egg white intoxication (the latter has been employed as a functional test for the presence of cortisone-like hormones in the rat). No significant impairment in pituitary-adrenal function was observed in pantothenic acid-depleted rats, as judged by the above indices.

LITERATURE CITED

CHEN, G., AND A. WICKEL 1952 Protective effect of steroids against acute egg-white toxicity in adrenalectomized rats. Endocrinology, *51:* 21.

COWGILL, G. R., R. W. WINTERS, R. B. SCHULTZ AND W. A. KREHL 1952 Pantothenic acid deficiency and the adrenals: some recent experiments and their interpretation. Internat. Zschr. Vitaminforsch., *23:* 275.

DOUGHERTY, T. F., AND A. WHITE 1944 Influence of hormones on lymphoid tissue structure and function. The role of the pituitary adrenotrophic hormone in the regulation of lymphocytes and other cellular elements of the blood. Endocrinology, *35:* 1.

DUMM, M. E., P. OVANDO, P. ROTH AND E. P. RALLI 1949 Relation of pantothenic acid to white blood cell response of rats following stress. Proc. Soc. Exp. Biol. Med., *71:* 368.

ERSHOFF, B. H. 1952 Nutrition and the anterior pituitary, with special reference to the general adaptation syndrome. Vitamins and Hormones, *10:* 79.

ERSHOFF, B. H., AND J. G. GAINES 1953 Comparative effects of total body and tail heating on the peripheral leukocyte count of the rat. Science (in press).

ERSHOFF, B. H., AND F. D. PARROTT, JR. 1953 Effects of B vitamin deficiencies on the leukocyte response to epinephrine and ACTH administration in the rat. J. Clin. Nutrition, *1:* 124.

HURLEY, L. S., AND A. F. MORGAN 1952 Carbohydrate metabolism and adrenal cortical function in the pantothenic acid-deficient rat. J. Biol. Chem., *195:* 583.

KREHL, W. A., R. W. WINTERS AND R. B. SCHULTZ 1952 Adrenal function in pantothenic acid deficiency. The National Vitamin Foundation, Inc., New York, N. Y., Nutrition Symposium Series No. 5, p. 39.

LONG, C. N. H. 1947 The relation of cholesterol and ascorbic acid to the secretion of the adrenal cortex. Recent Progress in Hormone Research, *1:* 99.

MORGAN, A. F. 1951 The effect of vitamin deficiencies on adrenocortical function. Vitamins and Hormones, *9:* 161.

NIEFT, M. L., AND H. J. DEUEL, JR. 1950 Studies on cholesterol esterases. I. Enzyme systems in rat tissues. J. Biol. Chem., *177:* 143.

PALMER, J. G., G. E. CARTWRIGHT AND M. M. WINTROBE 1951 The effects of ACTH and cortisone on the blood of experimental animals. Proc. 2nd Clin. ACTH Conf., *1:* 438; The Blakiston Co., Philadelphia, Pa.

PERRY, W. F., W. W. HAWKINS AND G. R. CUMMING 1953 Adrenal function in pantothenic acid deficiency. Am. J. Physiol., *172:* 259.

RECANT, L., D. M. HUME, P. H. FORSHAM AND G. W. THORN 1950 Effect of epinephrine on the pituitary adrenocortical system. J. Clin. Endocrin., *10:* 187.

REINHARDT, W. O., H. ARON AND C. H. LI 1944 Effect of adrenocorticotrophic hormone on leukocyte picture of normal rats and dogs. Proc. Soc. Exp. Biol. Med., *57:* 19.

ROE, H. J., AND C. A. KUETHER 1943 The determination of ascorbic acid in whole blood and urine through the 2,4 dinitrophenylhydrazine derivative of dehydroascorbic acid. J. Biol. Chem., *147:* 399.

SAMUELS, L. T. 1951 Nutritional factors and hormones in stress reactions. National Vitamin Foundation, Inc., New York, N. Y. Nutrition Fronts in Public Health, *3:* 142.

SAYERS, G., AND M. A. SAYERS 1948 The pituitary-adrenal system. Recent Progress in Hormone Research, *2:* 81.

SELYE, H. 1937 Studies on adaptation. Endocrinology, *21:* 169.

———— 1949 Effect of ACTH and cortisone upon an "anaphylactoid reaction." Canad. Med. Assn. J., *61:* 553.

———— 1950 Stress. Acta, Inc., Montreal, Canada.

———— 1951 Annual report on stress. Acta, Inc., Montreal, Canada.

WINTERS, R W., R. B. SCHULTZ AND W. A. KREHL 1952a The adrenal cortex of the pantothenic acid-deficient rat: eosinophile and lymphocyte responses. Endocrinology, *50:* 377.

———— 1952b The adrenal cortex of the pantothenic acid-deficient rat: carbohydrate metabolism. Ibid., *50:* 388.

THE SODIUM AND POTASSIUM REQUIREMENTS OF THE CHICK AND THEIR INTERRELATIONSHIP [1]

C. H. BURNS, W. W. CRAVENS AND P. H. PHILLIPS
Departments of Biochemistry and Poultry Husbandry, College of Agriculture, University of Wisconsin, Madison

THREE FIGURES

(Received for publication March 11, 1953)

The chemical, if not physiological, similarities between sodium and potassium make it logical to study simultaneously the nutritional behavior of these two minerals. Recent studies by Grunert, Meyer and Phillips ('50) showed that both sparing and antagonistic actions on the growth of rats could be demonstrated for sodium and potassium. High levels of either mineral were found to aggravate the effects of a low level of the other. A high level of sodium, however, was reported to lower the level of potassium required by weanling rats for early, rapid growth, although this effect subsequently disappeared. In extending these investigations to the nutrition of the chick, similar relationships have been found. The results of these and other experiments on the interrelationship between sodium and potassium are reported herein.

EXPERIMENTAL AND RESULTS

Straight run cross-bred (N.H. × S.C.W.L.) chicks from hens which were fed ration B1 of Robblee et al. ('48) were used in all experiments. The chicks were maintained in

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by a grant from the American Salt Manufacturers' Association.

electrically-heated batteries with raised screen floors and given water and the ration ad libitum. The basal ration was as follows, in per cent: dextrin, 55 to 58 (depending on the level of sodium and potassium salts); alcohol-extracted casein, 23; crude gelatin, 10; cystine, 0.3; soybean oil, 5. Vitamin supplements, in milligrams per kilogram, were: choline Cl, 2,000; B_{12} adsorbate,[2] 2,000; *i*-inositol, 1,000; riboflavin, 9; thiamine HCl, 6; Ca pantothenate, 20; niacin, 50; pyridoxine HCl, 8; α-tocopherol, 3; folacin, 2; 2-methyl-1,4-naphthoquinone, 0.5; biotin, 0.3. Vitamins A and D as fortified haliver oil were supplied weekly by dropper.

The minerals were added in the form of salt mixtures, "salts A" for diets without added sodium or potassium, and "salts B" for diets without added sodium or chloride. The composition of these mixtures and the amounts fed have been reported by Burns et al. ('52). They supplied calcium, 0.70%; phosphorus, 0.36% (23% casein raises this to 0.59%); potassium (when used), 0.73%; chloride, 0.51%; magnesium, 0.1%; iron, 22 mg %; manganese, 9 mg %; and iodine, zinc and copper at 3, 0.6 and 0.4 mg %, respectively.

The sodium and potassium content of the basal ration, when supplemented with salts A, was found by analysis to be 0.019% and 0.027%, respectively. Reagent-grade sodium and potassoum bicarbonates were added to the diet to supply the levels of sodium and potassium desired.

Whole blood was obtained from the chicks by heart puncture and analyzed for sodium and potassium by the method of Hald ('47), using the Perkins-Elmer flame photometer. Plasma was obtained by immediate centrifugation and analyzed without preliminary ashing.

Growth and mortality of chicks on various levels of sodium and potassium

To test the effect of the potassium level on the minimum requirement for sodium, the purified diet was supplemented

[2] Merck No. 3.

with salts A and the following levels of sodium: 0.02, 0.04, 0.10, 0.14, and 1.0%; at each level of sodium, the following levels of potassium were tested: 0.11, 0.15, 0.23, and 0.77%. The sodium levels were selected on the basis of a preliminary experiment in which growth of 250 to 300 gm was obtained

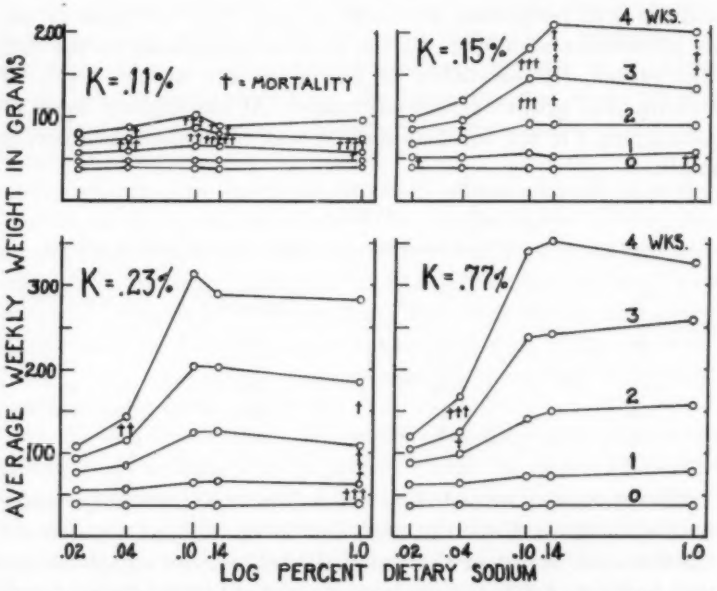

Fig. 1 Average weekly weight of chicks on varying levels of sodium and potassium. The experimental period was from the first to the 28th day.

in 4 weeks with diets containing 0.77% potassium and 0.06–0.14% sodium. Twelve-day-old chicks were placed on each diet, and individual weights were recorded weekly for 4 weeks. The weekly average weight and mortality of each group are shown in figure 1.

The poor growth and high mortality of the chicks fed 0.11% potassium indicated that this level of potassium was inadequate for growth and prevention of mortality at any level of sodium. With higher levels of potassium, growth at each of

the sodium levels tested increased progressively, but at each level of potassium, optimum growth appeared to occur with diets containing between 0.10 and 0.14% sodium. This level is taken as the minimum requirement for chicks fed purified diets and reaching 325 gm in weight in 4 weeks.

The levels at which mortality occurred show that both sodium and potassium are toxic if the other element is fed in insufficient amounts. As the level of potassium in the diet was raised, the mortality of chicks on the higher levels of sodium was progressively overcome. At the highest level of potassium, the low level of sodium was insufficient to prevent death.

TABLE 1

Protective effect of high potassium for chicks on diets high in sodium

Na	K	GAIN IN WEIGHT 12 DAYS	MORTALITY
%	%	*gm*	%
1.5	0.15	17	75
1.5	0.23	39	67
1.5	0.33	40	50
1.5	1.5	68	8

The protection afforded by potassium from mortality caused by high-sodium diets was observed in the second experiment. Chicks were supplied the purified basal ration supplemented with salts A, 1.5% sodium, and 4 graded levels of potassium. The results are shown in table 1. While growth increased with increasing dietary potassium, mortality remained high until the level of potassium was raised to that of sodium. The protection against sodium toxicity afforded by 1.5% potassium occurred despite the fact that the total amount of the bicarbonate salts constituted 9.3% of the diet.

To test levels of sodium and potassium not tested in the first experiment, the experimental procedure was repeated in the third experiment with sodium levels at 0.10, 0.18 and 0.32% of the diet, and at each level of sodium potassium was provided at 0.19, 0.33, 0.53, 0.77, 1.0 and 1.5% of the diet.

Because growth differences between these levels of sodium and potassium were not expected to be large, an attempt was made to standardize the experimental animals. All chicks used in this experiment were fed the purified basal diet supplemented with salts A, 0.10% sodium and 0.20% potassium for 18 days prior to the experimental period. On the basis of the weight and rate of gain of each chick, the most and least rapidly growing chicks were discarded, and the remainder were divided into 18 uniform groups of 9 chicks each. They were placed on the experimental diets on the 19th day, and individual weights were recorded one and two weeks thereafter.

The results are shown in figure 2, where growth is represented as the percentage gain in weight made during the experimental period. The data show that with diets containing 0.18% or more sodium, the maximum rate of gain was achieved with 0.33% potassium, but with diets containing 0.10% sodium, 1.0% potassium was required. Thus, between these levels of sodium and potassium, a mutual but slight sparing action was observed.

In the 4th experiment, the levels of sodium and potassium tested were confined to a narrow range in order to obtain more detail on the effect of one mineral on the requirement of the other. The period during which the animals were standardized was limited to the first 5 days after hatching. The purified basal ration was supplemented with salts A and the following levels of sodium: 0.08, 0.16, 0.33, and 0.60%. At each level of sodium, the following levels of potassium were supplied: 0.20, 0.30, 0.40, 0.55, and 0.77%. Seven chicks were placed on each diet on the 5th day. The average weight at 4 weeks of age for each group is shown in figure 3, where the data are plotted with respect both to the potassium level (fig. 3 a) and the sodium level (fig. 3 b) in the diet.

In figure 3 a it is apparent that, in this experiment, the requirement for potassium was 0.40%, and that maximum growth at this level of potassium was achieved with 0.16 to 0.33% sodium. The requirement for potassium appeared to

be increased if the level of sodium was raised or lowered beyond these limits, which suggested that there was an optimum ratio of these two minerals for growth, as well as optimum levels.

Figure 3 b shows that 0.33% sodium was required for optimum growth at the minimum requirement for potassium of 0.40%. There was no evidence in this experiment that levels

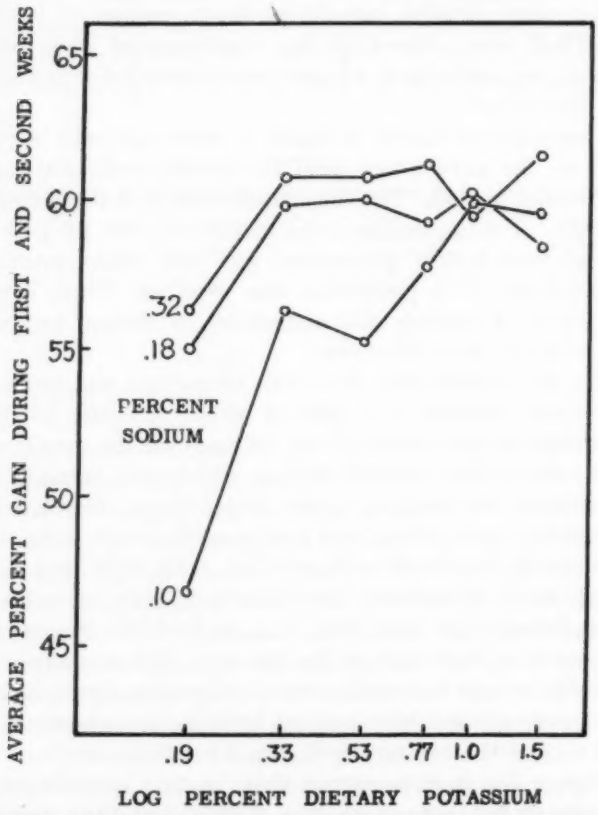

Fig. 2 Variation of per cent gain over a two-week period of chicks on varying levels of sodium and potassium. The experimental period was from the 18th to the 32nd day.

of sodium higher than 0.33% would permit the use of less than 0.40% potassium for the same rate of growth, although on the lower levels of potassium, higher levels of sodium did give some benefit.

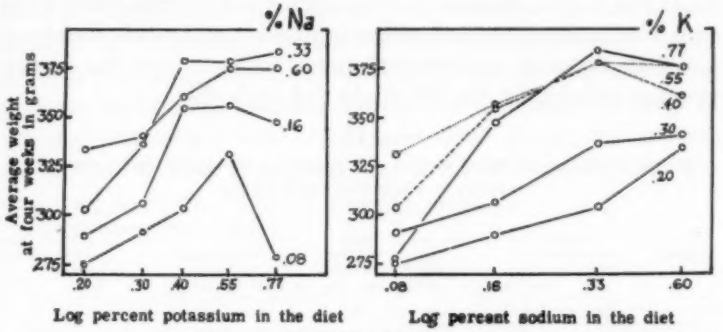

Fig. 3 Average weight at 28 days of chicks fed varying levels of sodium and potassium. The experimental period was from the 5th to the 28th day. a. Plotted with respect to the level of dietary potassium. b. Plotted with respect to the level of dietary sodium.

TABLE 2

Relative effect on growth of low sodium and low chloride diets

MILLIEQUIVALENTS/KG [1]		AVERAGE WEIGHT AT 4 WEEKS
Sodium	Chloride	
		gm
17.4	17.4	154
17.4	69.6	157
69.6	17.4	293
69.6	69.6	307

[1] 17.4 m.e. = 0.04% sodium = 0.062% chloride.
69.6 m.e. = 0.16% sodium = 0.246% chloride.

Comparison of effect of chloride- and sodium-deficient diets on growth

Because sodium is usually supplied as the chloride, an experiment was conducted to determine whether the above findings with regard to dietary sodium would be applicable to an equivalent level of dietary sodium chloride. The purified basal ration in this case was supplemented with salts B and

with sodium bicarbonate and ammonium chloride to provide sodium and chloride where used. The sodium-chloride-low diet contained 0.04% sodium and 0.06% chloride (17 milliequivalents/kilogram in each case), and levels of 0.16% sodium and 0.25% chloride (70 milliequivalents/kilogram) were added individually and together to provide a chloride-deficient, a sodium-deficient, and control diets. Table 2 gives the 4-week average weights of the 20 chicks fed each diet.

TABLE 3

Effect of dietary levels of sodium and potassium on sodium and potassium levels in whole blood and plasma

GROUP	DIET		AVE. WT. IN 4 WEEKS	MORTALITY 4 WEEKS	CONTENT IN MILLIEQUIV./100 ML			
					Whole blood		Plasma	
	K	Na			K	Na	K	Na
	%	%	gm	%				
				Experiment a				
1	0.13	0.02	86	45	4.1	7.1	0.77	10.4
2	0.13	0.12	96	15	3.7	9.9	0.36	13.1
3	0.33	0.02	151	75	3.8	6.2	0.87	10.4
4	0.33	0.12	216	5	3.5	9.5	0.44	13.4
				Experiment b				
1	0.11	0.02	71	58	3.0	7.0	0.43	10.6
2	0.11	0.35	73	50	3.3	9.1	0.28	12.9
3	0.23	0.02	100	0	3.3	7.6	0.87	11.2
4	0.23	0.35	259	17	3.3	10.1	0.64	13.4
5	0.77	0.02	101	0	3.6	6.6	1.05	10.9
6	0.77	0.35	315	0	3.5	9.0	0.61	15.2

The data show that, with diets containing 0.06% chloride, growth depended on the level of sodium in the diet. Raising the level of chloride to 0.25% had no effect. Similar results have been found with the laying hen (Burns et al., '52).

Effect of dietary sodium and potassium on the whole blood and plasma levels of sodium and potassium

Table 3 shows the results of whole blood and plasma analyses for sodium and potassium of chicks from two different experiments in which the dietary levels of sodium and potas-

sium were varied. The average 4-week body weights and total percentage mortality are also shown. Despite some inconsistency in the growth and mortality data, the trends in the sodium and potassium composition of the blood appear consistent, namely: (1) the increase of plasma and whole blood sodium in proportion to increasing dietary sodium is the most pronounced effect, (2) increasing the dietary sodium at any one level of potassium appears to decrease the level of plasma potassium, and (3) at any level of dietary sodium other than 0.02%, increasing the level of potassium in the diet increases the level of plasma sodium.

TABLE 4

Effect of growth rate on sodium requirement

TYPE OF DIET	MAXIMUM GROWTH ATTAINED	MINIMUM LEVEL OF SODIUM AFFORDING THIS GROWTH	EQUIVALENT LEVEL OF SODIUM CHLORIDE
	gm	%	%
Practical	280	0.09	0.22
Purified	325	0.10–0.14	0.25–0.35
Practical	355	0.25	0.60
Purified	385	0.30–0.35	0.75–0.85

DISCUSSION

The minimum requirement for sodium by the chick during the first few weeks of life appears to vary with the optimum rate of growth afforded by the experimental conditions. That this was the main factor in governing the minimum requirement was indicated by the results of a number of experiments in which the sodium intake was graded, as shown in table 4.

Since the requirement for chloride is less than 0.06%, the minimum requirement for sodium chloride is equivalent to that for sodium. Because some sodium chloride is present in most vegetable materials (Meyer et al., '50b), the allowance of 0.5% added sodium chloride recommended by the National Research Council ('50) may be considered adequate. Owing to the susceptibility of birds to sodium toxicity (Selye,

'43), the allowance should not exceed 1.0%, since some ingredients of natural diets, e.g., animal products, may themselves supply a considerable portion of the requirement.

The sodium requirement by the chick of 0.10 to 0.30% compares with 0.05% for the rat (Grunert et al., '50) and with 0.07% for the pig (Meyer et al., '50a).

The observation that chloride levels as low as 0.06% (equivalent to 0.10% sodium chloride) did not depress normal growth provided that the level of sodium was maintained at 0.16% (equivalent to 0.40% sodium chloride), showed that the requirement is lower than that for sodium, and that a salt deficiency is primarily a sodium deficiency. Similar observations of an apparently low chloride requirement were made with rats, where Osborne and Mendel ('18) found no decrease in growth when the chloride level was reduced to 0.035%. Greenberg and Cuthbertson ('42) found no striking, outward signs of deficiency with rats maintained for 15 weeks on a diet containing but 0.012% chloride. Urinary excretion was found to drop to less than 1% of normal a few hours after the rats were placed on the low-chloride diet, and increased bicarbonate and phosphate retention followed (Thacker, '43).

In regard to the requirement for potassium, it appears that the minimum requirement varies with the rate of growth. The most rapid rate of growth in these experiments was observed in experiment 4 (fig. 3). For maximum growth up to 4 weeks, the requirement for potassium was 0.40%. Figure 2 represents the growth of chicks fed the experimental diets from the 18th to the 32nd day, and the requirement appears to be met by 0.33% potassium, provided the level of sodium is 0.18%. In figure 1, results are shown for chicks fed the experimental diets from the first to the 28th day, but the rate of growth was not so rapid as in the 4th experiment. Since the over-all growth rate was slower, the chicks made a good growth increment when fed 0.23% potassium. These levels are higher than that reported by Gillis ('48), in which rapidly growing, White Leghorn male chicks fed a

cornstarch blood fibrin diet required 0.20 to 0.24% dietary potassium for maximum growth during the first 4 weeks.

Evidence of an antagonistic relationship between potassium and sodium was readily apparent in the growth retardation and mortality encountered when either element was supplied in considerable excess of the other. The antagonistic action of sodium on potassium has been found in the respiration of tissue slices (Buchanan et al., '49), of homogenates (Boyer et al., '43) and of bacteria (MacLeod and Snell, '48), as well as in the nutrition of animals. Antagonistic relationships are common among metabolites that are nearly but not quite identical; in the case of potassium and sodium, they are nearly identical as ions but probably differ in the degree to which they bind or form a complex with intracellular anions.

In contrast to the antagonistic action cited, the conditions under which a nutritional sparing action between the two can be observed do not indicate such a fundamental relationship. The sparing action of sodium on potassium in the diet of the rat disappears after two months of age, and it is produced by a diet which is toxic to the adult rat owing to the high sodium-to-potassium ratio. The sparing action in chicks, while not involving toxic levels of sodium, is detected by only small differences in growth and only when either sodium or potassium is barely limiting. The fact that increases in the level of dietary potassium led to higher plasma levels of sodium, and the fact that increases in the level of dietary sodium led to lower plasma levels of potassium, possibly because of increased retention within the cells, indicate that there may be conditions under which one mineral can promote the retention of the other.

SUMMARY

The minimum requirements for sodium and potassium by chicks fed semipurified diets for the first few weeks of life have been investigated, and both were found to vary with the rate of growth. The requirement for sodium was found

to lie between 0.10 and 0.30%, and that for potassium between 0.23 and 0.40%, the higher levels being required for maximum growth. The level of potassium appears to have no effect on the Na requirement, provided it is not a limiting factor.

The requirement for chloride was found to be less than 0.06%, and thus lower than its equivalent of the amount of sodium required.

Sodium and potassium were each found to be toxic if fed greatly in excess of the other, and this toxicity could be overcome by raising the level of the other element. Under certain conditions, a slight sparing action could also be observed between the two elements.

LITERATURE CITED

Boyer, P. D., H. A. Lardy and P. H. Phillips 1943 Further studies on the role of potassium and other ions in the phosphorylation of the adenylic system. J. Biol. Chem., *149:* 529.

Buchanan, J. M., A. B. Hastings and F. B. Nesbitt 1949 Effect of ionic environment on the synthesis of glycogen and total carbohydrate from pyruvate in liver slices. Ibid., *180:* 447.

Burns, C. H., W. W. Cravens and P. H. Phillips 1952 The requirement of breeding hens for sodium chloride. Poultry Sci., *31:* 302.

Gillis, M. B. 1948 Potassium requirement of the chick. J. Nutrition, *36:* 351.

Greenberg, D. M., and E. M. Cuthbertson 1942 Dietary chloride deficiency and alkalosis in the rat. J. Biol. Chem., *145:* 179.

Grunert, R. R., J. H. Meyer and P. H. Phillips 1950 The sodium and potassium requirements of the rat for growth. J. Nutrition, *42:* 609.

Hald, P. M. 1947 Flame photometer for measurement of sodium and potassium in biological materials. J. Biol. Chem., *167:* 499.

MacLeod, R. A., and E. E. Snell 1948 The effect of related ions on the potassium requirement of lactic acid bacteria. Ibid., *176:* 39.

Meyer, J. H., R. H. Grummer, P. H. Phillips and G. Bohstedt 1950a Sodium, chlorine, and potassium requirements of growing pigs. J. Animal Sci., *9:* 300.

Meyer, J. H., R. R. Grunert, R. H. Grummer, P. H. Phillips and G. Bohstedt 1950b The sodium, potassium, and chlorine content of feeding stuffs. Ibid., *9:* 153.

National Research Council 1950 Recommended nutrient allowances for poultry. Washington, D. C.

Osborne, T. B., and L. B. Mendel 1918 The inorganic elements in nutrition. J. Biol. Chem., *34:* 131.

ROBBLEE, A. R., C. A. NICHOL, W. W. CRAVENS, C. A. ELVEHJEM AND J. G. HALPIN 1948 Effect of hen diet and of chick diet on the need of chicks for an unidentified growth factor. Poultry Sci., *27:* 442.

SELYE, H. 1943 Production of nephrosclerosis in the fowl by sodium chloride. J. Am. Vet. Med. Assn., *103:* 140.

THACKER, E. J. 1943 Mineral composition of the albino rat as affected by chloride deficiency. J. Nutrition, *26:* 431.

THE VITAMIN B_{12} CONTENT OF EGG YOLKS AS INFLUENCED BY ORAL AND PARENTERAL ADMINISTRATION OF THE VITAMIN

J. V. HALICK, B. L. REID, C. L. BROWN AND J. R. COUCH
Departments of Poultry Husbandry and Biochemistry and Nutrition, Texas Agricultural Experiment Station, College Station

TWO FIGURES

(Received for publication March 2, 1953)

Couch and Olcese ('50) reported that the vitamin B_{12} content of chick liver was increased 5 times by supplementation of the diet with an animal protein factor (APF) concentrate. Furthermore, the injection of crystalline vitamin B_{12} (1 μg per bird per week) produced a three-fold increase in the B_{12} content of the livers.

Yacowitz et al. ('52) reported that the B_{12} content of egg yolks from hens fed a commercial breeder ration varied from 2.80 to 15.56 mμg of B_{12} per gram of yolk. Almost all of the vitamin B_{12} was found in the egg yolk, the white containing only negligible quantities. Halick and Couch ('51) reported earlier that both the feeding of a crude source or the injection of crystalline B_{12} (2 μg per hen per week) increased the B_{12} content of egg yolks approximately 5 times.

Surface-active agents have been reported to stimulate the growth of chicks (Ely, '51) and of pigs (Dyer, Davis and Cullinson, '52). Ely and Schott ('52) postulated that this growth stimulation might have been due to a more rapid assimilation of nutritional factors from the intestine due to the wetting properties of the detergents. However, other workers (Schendel and Johnson, '52; Voelker et al., '52) reported that neither the pig nor dairy calf showed a response to surfactants.

The present study was designed to determine the effect of feeding vitamin B_{12} to hens, and of injecting it, on the B_{12} content of egg yolks. It also appeared desirable to determine whether the addition of a surface-active agent to the ration might promote increased absorption of vitamin B_{12} from the gastrointestinal tract and result in a concurrent increase of B_{12} deposition in the egg.

EXPERIMENTAL

Four experiments were conducted during the course of the investigation herein reported. Single-Comb White Leghorn hens were used and the management of the birds was the same as that previously reported by Couch et al. ('48).

The basal diet was composed, in per cent, of 67.5 ground yellow corn, 25 soybean oil meal, 3 alfalfa leaf meal, 2.5 steamed bone meal, 1.5 oyster shell and 0.5 salt. In addition the ration was supplemented with 5 mg riboflavin, 7 mg calcium pantothenate, 2 mg pyridoxine hydrochloride, 50 mg niacin, 500 I.C.U. of vitamin D_3 and 4,000 I.U. of vitamin A per pound of feed. Ten grams of manganese sulfate were added to each 100 lb. of feed. The basal diet was modified slightly in experiments 3 and 4 by omitting the alfalfa leaf meal.

The first experiment was designed to determine the effect of feeding a high level of vitamin B_{12}, in the form of a crude concentrate, on the B_{12} content of egg yolks. Twenty-four pullets were divided into 6 groups of 4 birds each. Groups 1 through 6 were supplemented with 0, 50, 100, 250, 500 and 1,000 μg of B_{12} per kilogram of feed, respectively. A fuller's earth adsorbate of *Streptomyces griseus* fermentation mixture was used as the source of vitamin B_{12}. This product was stated by the manufacturer to contain 27.5 μg of B_{12} per gram. The yolks of all the eggs collected from each group on the first day of each week were pooled into one sample and assayed for vitamin B_{12}. The duration of this test was 9 weeks. Vitamin B_{12} was liberated from the yolks by autoclaving for 30 minutes in an acid pH according to the procedure of Halick and Couch ('51), and the autoclaved material was then as-

sayed microbiologically for the vitamin by the method of Skeggs et al. ('50).

In the second experiment 12 pullets were divided into three groups of 4 birds each. All birds were fed the basal ration and group 1 served as the control. The hens in group 2 received a single injection of 1 mg of crystalline B_{12} on the first day of the test, while each bird in group 3 was injected with 250 μg of B_{12} daily for the first 4 days of the test period. Injections were made in the pectoral muscles of the birds. Eggs were collected and assayed daily for a period of 16 days.

In the third experiment 4 groups of 6 pullets each were used. Each of the birds in group 2 received a daily injection of 250 μg of crystalline B_{12} for a period of 14 days, while those from group 3 were given orally the same level of B_{12} daily by pipette for the same period of time. Group 4 received the basal ration supplemented with 1,750 μg of B_{12} per kilogram of diet (equivalent to feeding 250 μg per bird per day). Eggs were collected daily and those laid on the 4th, 6th, 8th, 10th, 12th and 14th days of the experiment were assayed for B_{12}. At the end of the 14-day experimental period all groups were placed on the basal ration, and the eggs laid on the 21st, 28th, 35th and 42nd day were also assayed for B_{12}, to determine the length of time required for the B_{12} content of the yolks to return to a relatively normal level. Since it had been reported by Traina ('50) that the injection of a relatively large amount of a B_{12} concentrate was toxic to mice, it was deemed desirable here to determine what effects, if any, a high level of B_{12} in the egg would have on hatchability. Consequently, eggs laid on the third, 5th, 7th, 9th, 11th and 13th days of the experiment were incubated. Chicks hatched from these eggs were sacrificed at one day of age and the livers assayed for B_{12}. Vitamin B_{12} was liberated from the chick livers enzymatically by the method outlined by Couch and Olcese ('50) and the material then assayed microbiologically in the usual manner.

Experiment 4 was designed to determine the effect of a surfactant on the absorption of vitamin B_{12} from the intes-

tinal tract, as measured by the B_{12} content of the egg yolks. Thirty pullets were divided into 5 groups of 6 birds each. The hens in group 1 were fed the basal ration. Groups 2 and 3 were fed the basal ration supplemented with 30 μg B_{12} per kilogram of diet, and group 3 received in addition 0.2% of a surfactant.[1] The birds in group 4 received the basal ration supplemented with 1,750 μg B_{12} per kilogram of diet. The ration fed to group 5 also contained 1,750 μg B_{12} per kilogram, in addition to 0.2% of the surfactant. Eggs were collected daily for 14 days and those laid on the third, 5th, 7th, 9th, 11th and 13th days were assayed for B_{12}. Eggs not used for B_{12} analysis were incubated to determine the effect of this surfactant on hatchability.

RESULTS AND DISCUSSION

The results of the first experiment using a crude source of vitamin B_{12} show a decrease in the B_{12} content of egg yolks from hens fed the basal ration, which confirms an earlier report by Halick and Couch ('51). The feeding of 50 μg B_{12} per kilogram of ration maintained what was considered to be a relatively normal level of vitamin B_{12} (3.5 mμg/gm) in the egg yolks. The B_{12} content of the yolks increased as the level of the vitamin in the diet increased during the 9-week experimental period; however, there was little consistent difference in the B_{12} content of egg yolks from hens fed 500 μg and 1,000 μg B_{12} per kilogram. The value obtained for the B_{12} content of yolks (25.5 mμg/gm) from hens fed 1,000 μg B_{12} per kilogram was not as high as was expected, since other workers had reported B_{12} values as high as 15.56 mμg/gm in eggs from hens fed a commercial breeder ration (Yacowitz et al., '52; Lewis et al., '49). On the basis of the above observation it was not known whether the B_{12} in this adsorbate was fully utilized during the process of digestion; therefore, the pure vitamin was used in subsequent tests to eliminate the latter possibility.

[1] Polyoxylene oxide-substituted fatty amine.

The results of the second experiment (fig. 1) show conclusively that the injection of B_{12} into the hen markedly increased the B_{12} content of the yolks. Higher levels were

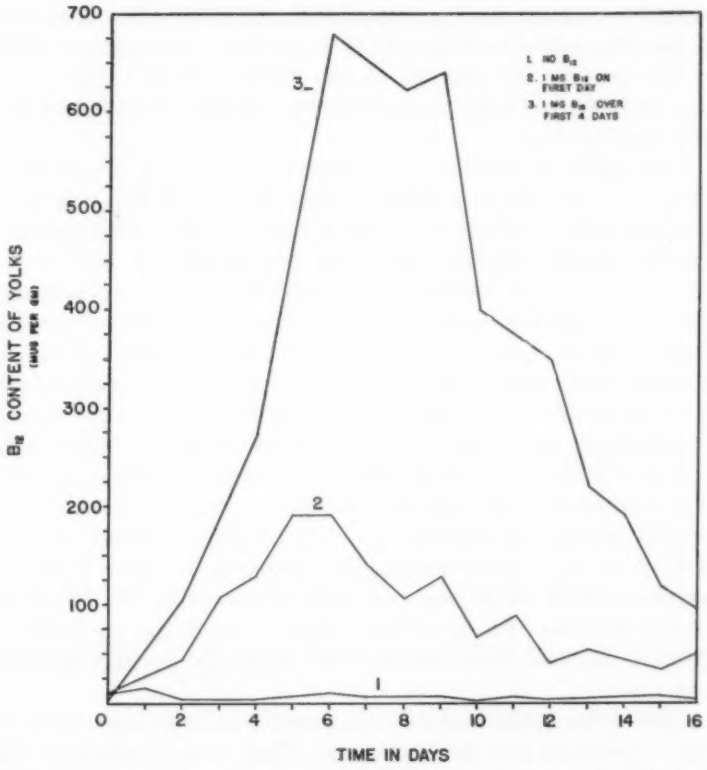

Fig. 1 Effect of intramuscular injection of vitamin B_{12} on the B_{12} content of egg yolks.

obtained when the vitamin was injected daily in small doses (group 3) than when the total amount was given in a single injection (group 2).

When 1 mg of B_{12} was injected in 4 daily doses of 250 µg each (group 3), there was an increase in the B_{12} content of

the egg yolks of about 70 times that of egg yolks from the uninjected group (group 1). The peak appeared on the 6th day after the first injection, with a rapid decline afterward. If it is assumed that the level of B_{12} in the yolks is directly related to the level of the vitamin in the blood, this decrease in the B_{12} content of the yolks is probably associated with an increase in the storage of the vitamin in the liver, and also an increase in the amount of the vitamin passing through the renal system.

The results of the third experiment (fig. 2) show the greater efficacy of parenteral administration of vitamin B_{12} over oral administration. Injection of the vitamin (group 2) increased the B_{12} content of the egg yolks approximately 100 times, but only a 6-fold increase was observed when the vitamin was administered orally (groups 3 and 4). These results suggest that there was a limited intestinal absorption or possible destruction of vitamin B_{12} in the gastrointestinal tract of the bird when the vitamin was ingested orally. These postulations are in agreement with the work of Barbee and Johnson ('51), who used radioactive B_{12} with the rat. The latter workers reported that when the vitamin was administered orally, approximately 92% of the radioactivity appeared in the feces within 72 hours, and when injected, approximately 95% was excreted in the urine in the same period of time. Furthermore, about 60% of the unabsorbed vitamin B_{12} had been decomposed, probably by bacterial action.

Under the conditions of this experiment the high level of B_{12} in the eggs had no deleterious effect on hatchability. The per cent hatchability of eggs from groups 1 through 4 of the third experiment was 93, 90, 91 and 84, respectively. The values for the B_{12} content of the livers of these chicks are given in table 1. The livers of the chicks from hens injected with 250 μg per day of B_{12} (group 2) showed B_{12} values which increased rapidly and paralleled those found in the egg yolks, while the chicks from the hens fed B_{12} orally (groups 3 and 4) showed quite small though definite increases in the B_{12}

content of the livers. Four weeks after the vitamin B_{12} administration was discontinued, the B_{12} level in the yolks in all groups had returned to normal.

It should be pointed out that the B_{12} values reported in this paper are relative and do not represent the maximum values obtainable, since no precaution was taken to protect

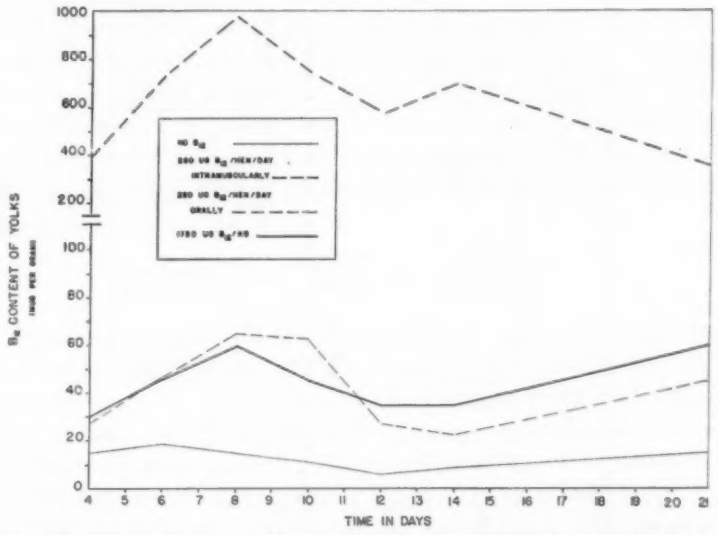

Fig. 2 Effect of intramuscular, oral and dietary administration of vitamin B_{12} on the B_{12} content of egg yolks.

B_{12}a from destruction (Soars and Hendlin, '51) during liberation of the vitamin from the samples by autoclaving. Assuming the presence of B_{12}a in the egg, and if the B_{12}a had been protected during autoclaving, then the B_{12} values reported in this study would have been higher, since both B_{12} and B_{12}a are equally active for *L. leichmannii* (Cooperman, Drucker and Tabenkin, '51). Preliminary observations from this laboratory support this point of view.

The results of feeding vitamin B_{12} with and without a surfactant in the 4th experiment are shown in table 2. The

TABLE 1

Effect of intramuscular, oral and dietary administration of vitamin B_{12} to hens on B_{12} content of chick livers

GROUP	TREATMENT	B_{12} CONTENT OF LIVERS [1]					
		3 days	5 days	7 days	9 days	11 days	13 days
1	None	41.7	68.0	42.8	48.0	45.2	50.3
2	250 μg B_{12}/hen/day, intramuscularly	94.0	357.0	653.8	331.6	480.0	665.8
3	250 μg B_{12}/hen/day, by pipette	57.5	83.3	94.5	—— [2]	72.0	74.3
4	1,750 μg B_{12}/kg of diet	35.5	46.0	56.0	118.0	127.6	117.7

[1] Millimicrograms per gram.
[2] Sample lost during autoclaving.

TABLE 2

The effect of feeding vitamin B_{12} with and without a surfactant on the B_{12} content of egg yolk

GROUP	SUPPLEMENT TO BASAL RATION	B_{12} CONTENT OF EGG YOLKS [1]							
		0	1 day	3 days	5 days	7 days	9 days	11 days	13 days
1	None	3.7	7.2	2.0	4.6	4.7	6.6	5.7	5.2
2	30 μg B_{12}/kg	3.0	5.4	10.4	11.3	12.4	19.1	15.2	16.5
3	30 μg B_{12}/kg + 0.2% surfactant	3.5	5.7	12.8	14.3	16.7	19.2	16.6	17.0
4	1,750 μg B_{12}/kg	5.5	4.6	24.4	22.2	37.4	46.2	41.6	38.3
5	1,750 μg B_{12}/kg + 0.2% surfactant	6.2	7.4	15.0	52.8	45.7	51.3	67.3	49.9

[1] Millimicrograms per gram.

feeding of the surfactant with a relatively low level of B_{12} (30 μg/kg, group 3) did not increase the absorption of B_{12} (as measured by the B_{12} content of the egg yolks) over that of the birds fed the same level of B_{12} alone (group 2). However, when the high level of B_{12} was fed with the surfactant (group 5) there was a decided increase in the B_{12} content of the egg yolks over that in eggs from hens fed the same level of B_{12} in the absence of the surfactant (group 4). This indicates that absorption of the vitamin was enhanced by the presence of the surfactant in the ration. However, these results cannot be interpreted as an explanation of the action of surfactants in increasing the growth of chicks, as far as B_{12} is concerned, since a much larger level of the vitamin was used here than is usually fed.

SUMMARY

Marked increases in the B_{12} content of egg yolks were obtained by the injection of high levels of the vitamin into the hen as compared with the B_{12} content of eggs from hens where the vitamin was fed. These differences indicate that there is limited absorption or possible destruction of vitamin B_{12} in the intestinal tract, or both, when it is ingested orally.

The presence of a surface-active agent and a high level of B_{12} in the ration resulted in an increase in the B_{12} content of the egg yolks over that in the yolks from hens fed the high level of B_{12} alone. These results suggest that absorption of the vitamin was enhanced, at least to a limited extent, by the presence of the surfactant. There was no apparent increase in the B_{12} content of eggs from hens fed the surfactant with a lower level of B_{12}.

Under the conditions of this experiment the high level of B_{12} observed in the eggs had no deleterious effects on hatchability. Similarly, the hatchability of eggs from hens fed the surfactant, with and without B_{12}, was normal.

ACKNOWLEDGMENTS

This work was supported in part by a grant-in-aid from the U. S. Industrial Chemicals Co., a Division of the National Distillers Products Corporation, New York, N. Y.

Crystalline vitamin B_{12} was supplied through the courtesy of the Veterinary Division, Merck and Co., Rahway, N. J.

LITERATURE CITED

BARBEE, K. W., AND B. C. JOHNSON 1951 Metabolism of radioactive vitamin B_{12} by the rat. Proc. Soc. Exp. Biol. Med., *76:* 720–721.

COOPERMAN, J. M., R. DRUCKER AND B. TABENKIN 1951 Microbiological assays for vitamin B_{12}: A cyanide enhancement effect. J. Biol. Chem., *191:* 135–141.

COUCH, J. R., W. W. CRAVENS, C. A. ELVEHJEM AND J. G. HALPIN 1948 Relation of carbohydrate to intestinal synthesis of biotin and hatchability in mature fowl. J. Nutrition, *35:* 57–72.

COUCH, J. R., AND O. OLCESE 1950 The vitamin B_{12} content of chick tissues as influenced by the diet. Ibid., *42:* 337–346.

DYER, I. A., T. J. DAVIS AND A. E. CULLINSON 1952 Certain surface active agents accelerate the rate of gain of pigs fed a corn-cottonseed meal ration. J. Animal Sci., *11:* 760.

ELY, C. M. 1951 Chick-growth stimulation produced by surfactants. Science, *114:* 523–524.

ELY, C. M., AND S. SCHOTT 1952 Surface active agents as growth stimulators in chick rations. 7th Distillers Feed Conference, Cincinnati, Ohio.

HALICK, J. V., AND J. R. COUCH 1951 Antibiotics in mature fowl nutrition. Proc. Soc. Exp. Biol. Med., *76:* 58–62.

LEWIS, U. J., U. D. REGISTER, H. T. THOMPSON AND C. A. ELVEHJEM 1949 Distribution of vitamin B_{12} in natural materials. Ibid., *72:* 479–482.

SCHENDEL, H. E., AND B. C. JOHNSON 1952 Effect of antibiotics, arsanilic acid, surfactants and sulfas on the growth of baby pigs fed synthetic diets. J. Animal Sci., *11:* 775.

SKEGGS, H. R., H. M. NEPPLE, K. A. VALENTIK, J. W. HUFF AND L. D. WRIGHT 1950 Observations on the use of *Lactobacillus leichmannii* 4797 in the microbiological assay of vitamin B_{12}. J. Biol. Chem., *184:* 211–221.

SOARS, M. H., AND D. HENDLIN 1951 Use of potassium cyanide in the *Lactobacillus leichmannii* assay for vitamin B_{12}. J. Bact., *62:* 15–17.

TRAINA, V. 1950 Toxicity studies on vitamin B_{12} in albino mice. Arch. Path., *49:* 278–279.

VOELKER, V., R. S. ALLEN, N. L. JACOBSON AND J. T. BLAKE 1952 *In vivo* (dairy calves) and *in vitro* studies with surface agents. J. Animal Sci., *11:* 779.

YACOWITZ, H., R. F. MILLER, L. C. NORRIS AND G. F. HEUSER 1952 Vitamin B_{12} studies with the hen. Poultry Sci., *31:* 89–94.

AGEWISE DIFFERENCES IN THE URINARY EXCRETION OF VITAMIN B_{12} FOLLOWING INTRAMUSCULAR ADMINISTRATION

DONALD M. WATKIN, CALVIN A. LANG, BACON F. CHOW AND NATHAN W. SHOCK

Section on Gerontology, National Heart Institute, National Institutes of Health, Bethesda, Maryland, and Baltimore City Hospitals, and the Department of Biochemistry, The Johns Hopkins University School of Hygiene and Public Health, Baltimore

ONE FIGURE

(Received for publication January 12, 1953)

In an earlier paper (Lang et al., '53), a difference in the rate of disappearance of vitamin B_{12} from the kidneys of young and old rats was demonstrated. In the present communication, measurements of the urinary excretion of this vitamin by healthy individuals of divergent ages are presented.

Interest in the role of water-soluble vitamins in the nutrition of the aged was stimulated in 1941 when Stephenson and his associates (Stephenson, Penton and Korenchevsky, '41) claimed that the addition of B vitamins and ascorbic acid to the dietary of older persons resulted in great improvement in "general vitality and vigor." A greater need for ascorbic acid (Rafsky and Newman, '43) and the existence of a state of "unsaturation" with respect to thiamine (Rafsky and Newman, '43) in the aged have also been claimed. However, in an extensive study Horwitt et al. (Horwitt, Liebert, Kreisler and Wittman, '48) could find no differences in the excretion of thiamine or riboflavin between young and old subjects. Furthermore, when test doses of various B vitamins were administered, no correlation was found between age and

urinary excretion in studies by Lossy et al. ('51) and Schmidt ('51a, b).

The urinary excretion of vitamin B_{12} was studied initially by Chow et al. ('50), who demonstrated both in healthy individuals and in those with pernicious anemia that B_{12} in massive doses (500 to 1,000 μg per individual) was excreted promptly and completely when administered parenterally but was excreted in negligible amounts when given orally. At lower levels of administration (20 to 75 μg per individual), however, Lang et al. ('52) found far lower percentages of administered doses excreted. Studies by other investigators are in substantial agreement with these observations (Chesterman, Cuthbertson and Pegler, '51; Jutton and Parsons, '51; Sokoloff, Sanneman and Beard, '52).

The present study was designed to examine the relation between the age of the subject and the urinary excretion of parenterally administered vitamin B_{12} by males.

EXPERIMENTAL

Subject selection

Adult males in different age groups comprised the experimental populations. In the first study, 19 healthy young student volunteers were compared with 16 patients over 65 years of age drawn from the population of the chronic division of the Baltimore City Hospitals. Subjects in the older group met the requirements of being over 65 years of age, ambulatory, continent, cooperative and free from clinically apparent liver disease as judged by history, physical examination and, in most instances, one or more laboratory tests,[1] and free from diabetes mellitus as judged by history, physical examination and by absence of glucosuria and a fasting blood sugar level below 100 mg %. Patients with renal disease, as

[1] These consisted of cephalin flocculation, thymol turbidity, serum bilirubin, total protein, albumin and globulin, alkaline phosphatase, B.S.P. excretion and urinary urobilinogen. These tests were performed as clinical diagnostic procedures. The analytical work was performed by the clinical laboratory of the Baltimore City Hospitals, whose cooperation is sincerely appreciated.

indicated by history or clinical and laboratory findings, were excluded from the study.

In the second experiment, three groups representing youth, middle age and old age, of 24 subjects each, were drawn from residents of a single institution. The young subjects were staff members and house officers of the Baltimore City Hospitals. The middle and old age subjects were chosen from the wards of the Baltimore City Hospitals. All met the criteria of the first study.

All of the middle and old age subjects had been residents of the Hospital for at least three months prior to the test. Over half of the subjects had been in the Hospital for more than two years. The diet received during this period was estimated at 1,800 to 2,200 cal./day; 9 to 12 gm N/day; 1.0 to 1.1 gm phosphorus; 40 to 50 mEq. of K; 200 to 250 gm carbohydrate, and 50 to 70 gm fat per day. Meat, fish or eggs (100 gm/day) as well as milk (100 to 200 ml/day) were included in the diet.

Experimental design

Each subject was given a single intramuscular injection of vitamin B_{12}.[2] Subgroups of 4 to 6 subjects, chosen at random, in each age category received dosages of 20, 30, 50 or 75 μg. Urine was collected for a 24-hour period prior to injection and from zero to 8 hours and 8 to 24 hours following injection.

The young individuals maintained their usual levels of activity throughout the experimental period. The others were housed in a special metabolism unit and were under continuous observation.

All subjects were encouraged to increase fluid intake to insure adequate urine output. Urines were collected in bottles containing 10 ml of toluene as a preservative. The volume of each urine specimen was measured and 15-ml aliquots were

[2] Supplies of an aqueous solution of crystalline vitamin B_{12} (without preservative) were furnished through the courtesy of Merck and Co., Inc.

stored at 5°C. until assayed for vitamin B_{12} activity by the technique of Skeggs et al. ('50), utilizing *Lactobacillus leichmannii* 4797 as the test organism. Experiments indicated that the assay permitted recovery of added vitamin with an error

TABLE 1

The urinary excretion of vitamin B_{12} in 107 subjects
(Studies I and II)

STUDY	GROUP	DOSE B_{12}	NO. OF SUBJECTS	MEAN AGE	MEAN WEIGHT	MEAN EXCRETION OF B_{12}
		μg		*years*	*kg*	*μg/24 hours*
I	Young	20	5	33.8 ± 4.5[1]	64.5 ± 3.5[1]	1.38 ± 0.15[1]
		30	5	28.3 ± 1.0	76.3 ± 4.2	1.86 ± 0.39
		50	5	29.4 ± 1.8	76.7 ± 4.0	10.4 ± 2.10
		75	4	27.4 ± 0.7	74.0 ± 1.1	23.5 ± 4.79
	Old	20	4	77.1 ± 2.9	61.7 ± 3.7	0.31 ± 0.08
		30	4	74.5 ± 4.0	68.9 ± 6.3	1.10 ± 0.13
		50	4	77.0 ± 5.2	63.0 ± 8.4	4.42 ± 1.04
		75	4	76.8 ± 1.8	65.5 ± 8.4	10.8 ± 1.68
II	Young	20	6	27.1 ± 0.9	69.4 ± 3.4	1.72 ± 0.37
		30	6	28.9 ± 1.0	74.7 ± 3.9	2.00 ± 0.76
		50	6	27.9 ± 0.6	72.4 ± 3.7	14.7 ± 1.05
		75	6	28.2 ± 0.9	78.6 ± 3.4	26.7 ± 3.03
	Middle	20	6	62.8 ± 1.02	56.0 ± 2.8	0.80 ± 0.14
		30	6	58.8 ± 1.06	65.8 ± 1.6	2.35 ± 0.67
		50	6	62.1 ± 1.62	61.0 ± 2.5	9.57 ± 1.41
		75	6	61.8 ± 1.28	62.1 ± 3.4	22.3 ± 2.93
	Old	20	6	81.3 ± 2.3	54.1 ± 2.6	0.42 ± 0.13
		30	6	79.3 ± 1.3	59.3 ± 6.9	1.44 ± 0.37
		50	6	83.3 ± 2.2	63.3 ± 6.0	8.58 ± 3.78
		75	6	82.5 ± 1.8	57.2 ± 6.3	17.0 ± 2.15

[1] ± Standard deviation of the mean.

of ± 20%. The minimum amount of B_{12} determinable by this method was 0.010 mμg.

RESULTS

None of the urine samples collected during the 24 hours preceding injection showed measurable B_{12} activity. Following administration of the test dose, 6 out of a total of 107

samples of the 8-hour collections contained less than 95% of the total activity excreted during the entire period of collection. For this reason, the activities measured in the 8- and 16-hour samples have been combined and reported as 24-hour excretions in the tabulations. These values have also been used for the statistical calculations. Table 1 summarizes the data from both studies.

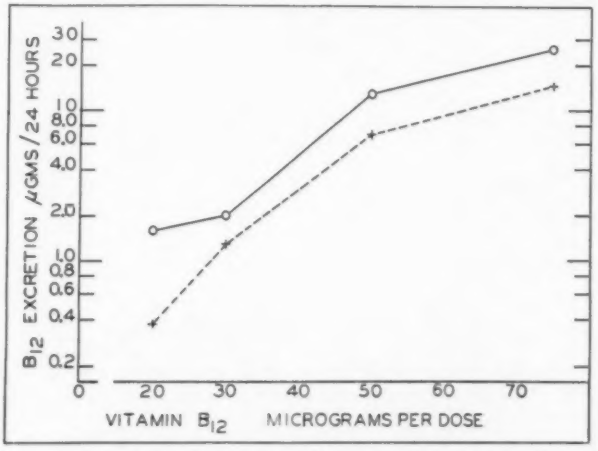

Fig. 1 A semi-log plot relating the urinary excretion of vitamin B_{12} to test doses at 4 levels in young (solid line) and old (interrupted line) groups from studies I and II combined.

The mean excretion values at all dose levels, in both studies, show that the old subjects excreted less of the test dose than the young ones. In study II, with the exception of the data of the 30-µg dosage level, there was a consistent downward trend of mean excretion with increasing age for the three age groups. It seems likely that the higher excretion of the middle-aged group at the 30-µg dose level is an artifact of the experiment.

In figure 1 are plotted curves relating the urinary excretion of vitamin B_{12} to the test dose for the young and old

groups, combining the data from both studies. The data, plotted on the logarithmic scale, show that the amount of B_{12} excreted increased progressively with increasing dose and that at all dose levels the aged subjects excreted less of the administered vitamin than did the young.

An analysis of variance was performed on the data from each experiment to test the statistical significance of the effects of both age and dose level on the excretion of vitamin B_{12}. The analysis was performed after a logarithmic transforma-

TABLE 2

Analyses of variance [1]

STUDY	SOURCES OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	"F" RATIO
I	Ages	1	0.3175	32.40 †
	Doses	3	0.7900	80.61 †
	Age × dose interaction	3	0.0227	2.32 ‡
	Individual variation	27	0.0098	..
II	Ages	2	0.5126	4.04 †
	Doses	3	9.0005	70.95 †
	Age × dose interaction	6	1.0164	1.34 ‡
	Individual variation	60	0.1268	..

[1] These analyses were completed following a logarithmic transformation of the original excretion data. For study I, the analysis was calculated as if the numbers of subjects at each dose level for each age group were equal.

† = significant ‡ = not significant

Significance is tested at the 0.05 level; i.e., a value of "F" is considered significant if a value as large or larger could have occurred 5% of the time by chance alone.

tion of the excretion data in order to minimize differences in standard deviations of the measurements made at different dose levels. The results of the analysis (table 2) show that in both studies the effects of age and dose were significant. Since the age × dose interaction was not significant, it may be concluded that there is no evidence that any given dose level acts differently at different ages.

DISCUSSION

These studies present evidence only on the urinary excretion of vitamin B_{12} administered parenterally in young and

old. If the assumption is made that the excretion of B_{12} is proportional to renal function, it follows that the urinary loss of B_{12} per unit of time will be reduced in elderly subjects, since renal function has been shown to diminish progressively with advancing age (Davies and Shock, '50). However, since only a small fraction of the administered dose appeared in the urine, it is apparent that a much larger fraction was not excreted and, therefore, that age differences in non-renal pathways of disappearance of the vitamin probably also exist. These studies do not provide data with which to assess the nature of these differences.

Although the urinary excretion of a vitamin following a test dose has been used as an index of nutritional status of the subject with respect to that vitamin, it cannot be inferred that the older subjects in this study were suffering from a B_{12} deficiency. All subjects had received diets providing adequate B_{12} for many months. Although the incidence of achlorhydria increases with advancing age (Bloomfield and Polland, '33) there is no experimental evidence (Ivy and Grossman, '52) that a decrement in "intrinsic factor," and thus in B_{12} absorption, occurs concurrently.

The general dietary intakes of the middle aged and old subjects of study II were estimated to be essentially similar. It has also been shown that the excretion of intramuscular B_{12} in humans is not influenced significantly by the prior dietary intake of the vitamin (Chow et al., '50). Therefore, differences, if any, in the prior dietary intake of vitamin B_{12} in our subjects cannot explain the observed agewise differences in excretion.

Despite uncertainty as to the mechanisms underlying the observed differences in B_{12} excretion, there can be no doubt that differences betwen young and old exist, since they are susceptible of direct measurement and are of considerable magnitude. In the absence of an explanation, it would be premature to infer that these findings justify the conclusion that old individuals necessarily suffer from a B_{12} deficiency,

or, as a corollary, that B_{12} would be a significantly valuable supplement to their dietary.

SUMMARY

1. The urinary excretion of vitamin B_{12} following its intramuscular administration in crystalline form was studied at 20, 30, 50 and 75 μg dose levels in groups of male subjects, aggregating 107 individuals in widely separated age categories.

2. In study I, a young group averaging 29.7 years excreted significantly more B_{12} than did an old group averaging 76.4 years.

3. In study II, an orderly and significant decrement in B_{12} excretion was observed from young (28.0 years) to middle (61.4 years) to old (81.6 years) groups.

4. The decrement in B_{12} excretion with age parallels decrements in renal function with age. However, so small a fraction of administered B_{12} appears in the urine that the observed differences cannot be attributed to renal factors alone.

ACKNOWLEDGMENTS

The authors are indebted to members of the house staff of the Baltimore City Hospitals, to the staff of the Section on Gerontology, and to the students and staff of the School of Hygiene and Public Health who participated in this study. Our thanks are also due members of the nursing staff of the Baltimore City Hospitals for their supervision of the aged patients and to Mrs. Shanley Davis for her performance of the microbiological assays. We gratefully acknowledge the assistance of Dr. Max Halperin in formulating the appropriate statistical analysis. We appreciate also the helpful suggestions of Dr. Milton Landowne.

LITERATURE CITED

BLOOMFIELD, A. L., AND W. S. POLLAND 1933 Gastric Anacidity; Its Relation to Disease. The Macmillan Co., New York, N. Y.

CHESTERMAN, D. C., W. F. J. CUTHBERTSON AND H. F. PEGLER 1951 Vitamin B_{12} excretion studies. Biochem. J., *48:* 1.

CHOW, B. F., C. A. LANG, R. DAVIS, C. L. CONLEY AND C. ELLICOTT 1950 The appearance of vitamin B_{12} activity in urine after oral and intramuscular administration to man. Bull. Johns Hopkins Hosp., *87:* 156.

DAVIES, D. F., AND N. W. SHOCK 1950 Age changes in glomerular filtration rate, effective renal plasma flow and tubular excretory capacity in adult males. J. Clin. Invest., *29:* 496.

HORWITT, M. K., E. LIEBERT, O. KREISLER AND P. WITTMAN 1948 Investigations of human requirements for B-complex vitamins. Bull. Natl. Res. Council, *116.*

IVY, A. C., AND M. I. GROSSMAN 1952 Cowdry's Problems of Aging, Biological and Medical Aspects. Chapter 20, Digestive System, p. 421, 3rd ed.

JUTTON, D. R., AND H. T. PARSONS 1951 Excretion of vitamin B_{12} by human subjects. Fed. Proc., *10:* 203.

LANG, C. A., AND B. F. CHOW 1952 Retention of vitamin B_{12} after administration to subjects of different ages. Fed. Proc., *11:* 88.

LANG, C. A., D. M. GLEYSTEEN AND B. F. CHOW 1953 The disappearance of radioactivity from tissues of rats of different ages after subcutaneous administration of radiovitamin B_{12}. J. Nutrition, *50:* 213.

LANG, C., R. A. HARTE, C. L. CONLEY AND B. F. CHOW 1952 Retention of crystalline vitamin B_{12} by healthy male individuals following intramuscular injection. Ibid., *46:* 215.

LOSSY, F. T., G. A. GOLDSMITH AND H. P. SARETT 1951 A study of test dose excretion of five B-complex vitamins in man. Ibid., *45:* 213.

RAFSKY, H. A., AND B. NEWMAN 1941 Vitamin C studies in the aged. Am. J. Med. Sci., *201:* 749.

———— 1943 Vitamin B excretion in the aged. Gastroenterology, *1:* 737.

SCHMIDT, V. 1951a The excretion of pantothenic acid in the urine in young and old individuals. J. Geront., *6:* 132.

———— 1951b The excretion of pantothenic acid in patients with achromotrichia and alopecia. Ibid., *6:* 369.

SKEGGS, H. R., H. M. NEPPLE, K. A. VALENTIK, J. W. HUFF AND L. D. WRIGHT 1950 Observations on the use of *Lactobacillus leichmannii* 4797 in the microbiological assay of vitamin B_{12}. J. Biol. Chem., *184:* 211.

SOKOLOFF, M. F., E. H. SANNEMAN, JR., AND M. F. BEARD 1952 Urinary excretion of vitamin B_{12}. Blood, *7:* 243.

STEPHENSON, W., G. PENTON AND V. KORENCHEVSKY 1941 Some effects of vitamin B and C on senile patients. Brit. Med. J., *2:* 839.

THE EFFECT OF HEAT ON THE NUTRITIVE VALUE OF MILK PROTEINS AS INFLUENCED BY WATER AND FAT [1]

LAWRENCE J. SCHROEDER, MICHAEL IACOBELLIS, HELEN LEES AND ARTHUR H. SMITH

Department of Physiological Chemistry, Wayne University College of Medicine, Detroit

FIVE FIGURES

(Received for publication February 24, 1953)

In a previous paper experimental data were described which show that the heat employed in the commercial manufacture of evaporated and dried milk produces no nutritive damage to the contained protein when assayed by the nitrogen balance method (Schroeder, Iacobellis and Smith, '53). In 1952 Hodson, in carrying out investigations by the rat repletion method of protein evaluation, demonstrated that the sterilization of evaporated milk did not reduce the nutritional value of its protein, thus substantiating the results of our previous paper ('53) and those of Whitnah ('43). However, earlier studies (Davis, Rizzo and Smith, '49; Mader, Schroeder and Smith, '49; Schroeder, Stewart and Smith, '51) demonstrated that, when dry lactalbumin is autoclaved, the digestibility of the protein is so altered that its over-all nutritive value is greatly diminished. It became necessary to account for these differences in experimental results with milk protein. The

[1] Some of the data in this paper are taken from the dissertation submitted by Michael Iacobellis for the degree of Doctor of Philosophy, Wayne University. Preliminary reports of the work were presented before the Michigan Academy of Science at Ann Arbor, April 11, 1952, and the American Institute of Nutrition at New York, April 16, 1952.

most obvious difference between the two sets of experiments is that the protein was heated in the presence of water (fluid milk) in the more recent study, whereas heat was applied to the dry protein in the earlier investigations. Experiments were therefore planned to determine the part which water plays in protecting milk proteins from damage by heat. The study was planned to include also the influence of fat upon this reaction.

EXPERIMENTAL

Attempts to secure purified, non-denatured lactalbumin in quantity to be used in the preparation of a "synthetic milk" were unsuccessful. Therefore, a commercially available nonfat dry milk solid product [2] was chosen as the source of protein in the experimental diets. This material formed a homogeneous dispersion in water and could be reconstituted with water to any desired protein content. The reconstituted product (3.5% protein), unautoclaved, served as the control milk. Samples of the dried skim milk (35% protein) were dry autoclaved at 15 pounds' pressure for 30 minutes. In addition, samples reconstituted to protein levels of 3.5 and 17.5% were similarly heat-treated. These various treated and processed milks were added to the protein-free basal ration (Schroeder, Iacobellis and Smith, '53) to a total protein level of 10%.

The biologic evaluation of the nutritive value of the milk proteins after the foregoing treatments was made on the basis of digestibility and of nitrogen balances, using three adult female mongrel dogs (10.5, 8.0 and 14.8 kg). The method of protein evaluation was that of Murlin and associates ('41) as described in our previous paper (Schroeder, Iacobellis and Smith, '53).

The average data (fig. 1) for nitrogen balance of the three dogs, as they were alternately fed the control (unheated) and the various experimental milks, show that the dried skim milk, which had been autoclaved in the dry state, failed to maintain

[2] "Starlac," kindly furnished by the Borden Company, Detroit, Michigan.

a positive nitrogen balance, whereas, when the dogs were fed the ration whose protein was provided exclusively by the milks which were reconstituted before heating, a positive nitrogen balance was observed. The detailed data are shown in table 1. The average digestibility, biological value and nutritive index for the contained protein are shown in table 2; the evident nutritional superiority of the milks reconstituted before autoclaving strongly suggests that the presence of water prevents the decrease in the nutritive value of the proteins when

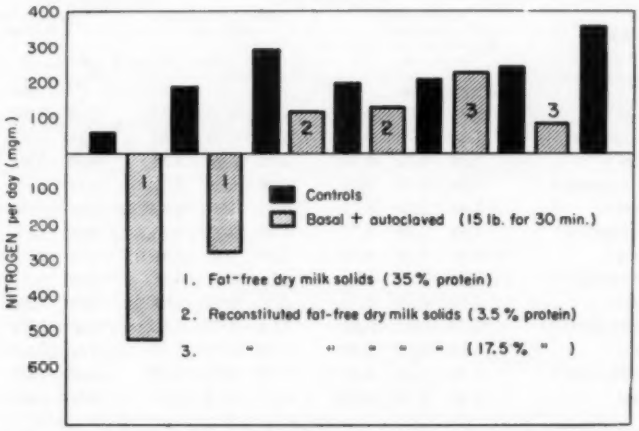

Fig. 1 Average nitrogen balances in dogs fed heated and unheated milk samples.

autoclaved in the presence of the other non-fat solids of milk. In these experiments involving the nitrogen balance on dogs, little difference was observed between the milk sample reconstituted to a protein level of 3.5% and that reconstituted to a level of 17.5% protein; the limiting amount of water beyond which the protein would show heat damage under the present experimental conditions was, therefore, not determined.

In order to secure evidence on this latter point, a series of *in vitro* tests were carried out. Milk samples made from the

dried skim milk by various degrees of reconstitution with water were autoclaved as before, and the amino nitrogen released by crystalline trypsin or chymotrypsin was quantitatively determined. The possible influence of fat was evaluated by a comparison of the non-fat dried milk solids and whole milk powder, similarly treated.

TABLE 1

Food, urinary and fecal nitrogen data on dogs fed heated and unheated milk samples

DIET	TOTAL FOOD N DOG NO.			TOTAL URINARY N DOG NO.			TOTAL FECAL N DOG NO.		
	1	2	3	1	2	3	1	2	3
		gm.			gm.			gm.	
Control [1]	5.84	5.59	6.70	4.59	4.28	5.54	1.18	0.93	0.99
Experimental [2]	7.04	6.73	8.08	6.77	6.38	8.65	2.18	1.57	2.60
Control [1]	5.84	5.59	6.70	4.62	3.80	5.34	0.53	0.89	1.23
Experimental [3]	7.04	6.73	8.08	6.02	6.24	6.27	2.12	2.06	2.51
Control [1]	5.84	5.59	6.70	4.11	4.87	4.23	1.16	0.59	0.50
Experimental [3]	7.64	7.09	8.72	4.98	5.96	5.51	1.75	1.56	2.24
Control [1]	5.84	5.59	6.70	3.53	4.42	5.46	1.21	0.98	0.74
Experimental [3]	7.73	7.18	8.83	5.21	4.96	6.88	1.85	1.30	2.03
Control [1]	5.84	5.59	6.70	3.64	3.95	5.43	0.86	1.28	1.08
Experimental [4]	7.78	7.44	8.92	5.94	5.67	5.88	1.33	0.75	1.82
Control [1]	5.84	5.59	6.70	4.39	4.29	5.09	0.66	0.52	0.98
Experimental [4]	7.78	7.44	8.92	5.52	6.34	6.47	1.95	1.25	1.56
Control [1]	5.84	5.59	6.70	3.92	4.20	5.67	0.80	0.47	0.31

[1] Basal diet + unautoclaved reconstituted (3.5% protein) fat-free dry milk solids — three days.

[2] Basal diet + autoclaved (35% protein) fat-free dry milk solids — 4 days.

[3] Basal diet + autoclaved reconstituted (3.5% protein) fat-free dry milk solids — 4 days.

[4] Basal diet + autoclaved reconstituted (17.5% protein) fat-free dry milk solids — 4 days.

The milk samples to be used for enzymatic hydrolysis were prepared from both whole milk powder and fat-free dry milk solids in the following manner. In the first experiment, both of these materials were subjected to "dry autoclaving" at 15 pounds' pressure for 30 minutes. A modification of the methods of Lowry and Thiessen ('50) and Melnick and Oser ('49) was used in the proteolysis experiments. The equivalent of

2 gm of the contained protein was suspended in 50 ml of water, 30 mg of crystalline enzyme, trypsin,[3] or chymotrypsin [4] per gram of protein added, and the whole made up to a volume of 100 ml with 0.2 M phosphate buffer. The pH was adjusted to 8.6 with sodium hydroxide in each case. Aliquots of the protein digest were removed at various time intervals

TABLE 2

Average nutritional values of milk protein

PROTEIN SOURCES	DIGESTIBILITY [1]	BIOLOGICAL VALUE [2]	NUTRITIVE INDEX [3]
	%	%	%
Autoclaved [4] fat-free dry milk solids (35% protein)	86.6	90.6	78.5
Autoclaved [4] reconstituted fat-free dry milk solids (3.5% protein)	93.3	103.9	97.0
Autoclaved [4] reconstituted fat-free dry milk solids (17.5% protein)	94.9	99.8	94.7

[1] Digestibility (per cent) $= \frac{\text{Food N absorbed}}{\text{Total food N}} \times 100.$

Food N absorbed = total food N — (experimental fecal N — control fecal N).

[2] Biological value (per cent) $= \frac{\text{Food N absorbed} - (\text{experimental urinary N} - \text{control urinary N})}{\text{Food N absorbed}} \times 100.$

[3] Nutritive index = biological value × digestibility.

[4] Fifteen pounds' pressure for 30 minutes.

(figs. 2, 3, 4 and 5). Amino nitrogen determinations were made by the method of Pope and Stevens ('39), in duplicate.

In order to study the effect of the presence of water, other samples of either whole milk powder or fat-free dry milk solids were reconstituted to give varying protein concentrations and autoclaved as before. Analyses by the Kjeldahl method showed no change in total nitrogen content due to the heating process. Two-gram protein aliquots were removed as be-

[3] Trypsin, 2 × crystallized (approximately 50% $MgSO_4$ and 8% P.N.; specific activity 0.17), Worthington Biochemical Laboratory, Freehold, New Jersey.

[4] Chymotrypsin, salt-free, crystallized (bovine pancreas; 12.2% N; potency 0.04 P.U.), Armour and Company, Chicago, Illinois.

fore and subjected to proteolysis as previously described. The unautoclaved reconstituted milks (whole milk powder and nonfat dry milk solids — 3.5% protein) served as control samples in the digestion with trypsin and chymotrypsin.

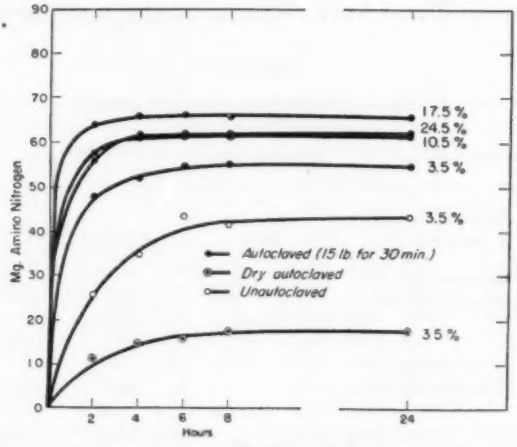

Fig. 2 Enzymatic digestibility of the protein of fat-free milk solids as measured by amino nitrogen liberation by trypsin.

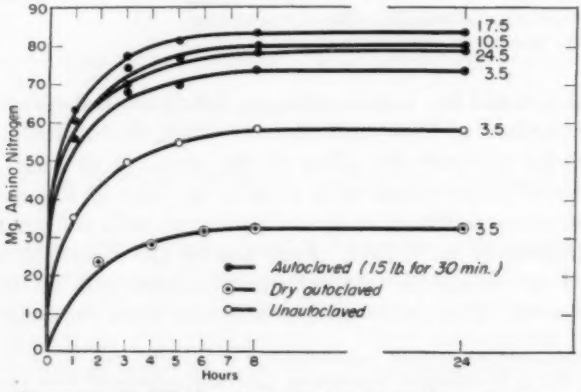

Fig. 3 Enzymatic digestibility of the protein of fat-free milk solids as measured by amino nitrogen liberation by chymotrypsin.

The results of the *in vitro* digestion experiments are shown in the accompanying graphs. In figures 2 and 3 it is seen that when dried skim milk powder as such (containing 35% protein) is autoclaved for 30 minutes at 15 pounds' pressure,

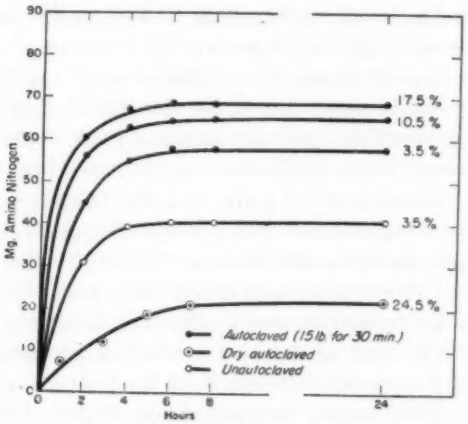

Fig. 4 Enzymatic digestibility of the protein of whole milk powder as measured by amino nitrogen liberation by trypsin.

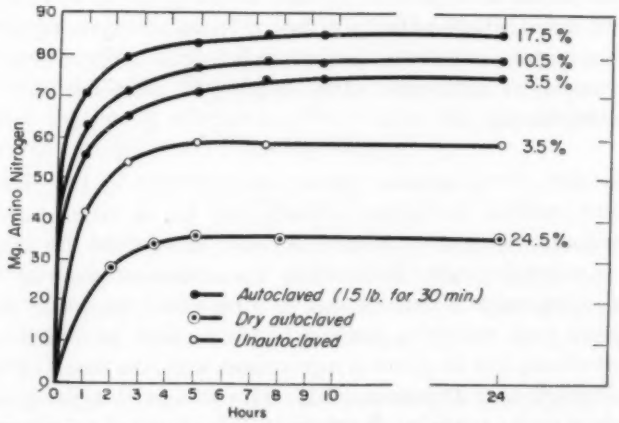

Fig. 5 Enzymatic digestibility of the protein of whole milk powder as measured by amino nitrogen liberation by chymotrypsin.

less than 20 mg of amino nitrogen are liberated from 2 gm of protein by trypsin and only about 30 mg by chymotrypsin. On the other hand, roughly three times more amino nitrogen is liberated by these enzymes from the proteins after similar heat treatment provided the dried skim milk had been previously reconstituted to levels of 3.5, 10.5, 17.5 or 24.5% protein. The curves representing the digestion of the samples variously diluted are closely grouped, so that it appears that as little added water as is represented by the 24.5% level of protein serves to protect the protein against heat injury. Of particular interest is the difference in *in vitro* digestibility between the unheated dried milk and the heated reconstituted samples; the results have consistently demonstrated that under the experimental conditions of this study the *in vitro* digestibility of the proteins of dried skim milk after reconstitution with water is enhanced by the heat treatment employed.

It is possible that the fat in the whole milk powder exerted a protective action against injury of the protein by heat. Earlier studies (Schroeder, Iacobellis and Smith, '53) of the nitrogen balance of dogs, in which whole milk powder was used, allowed no conclusion to be drawn as to the influence of fat. Furthermore, as is seen in figures 4 and 5, the *in vitro* digestion of dried whole milk by either trypsin or chymotrypsin is approximately equal to that of dried skim milk when they are compared after the same degree of reconstitution and heat treatment.

DISCUSSION

The data in the present paper, secured both by the nitrogen balance method on intact animals and by *in vitro* digestion experiments with crystalline enzymes, show that the diminution in nutritive value suffered by the contained proteins when dried skim milk is autoclaved, is prevented by prior reconstitution with water to protein levels as high as 24.5%. Our observations are in general agreement with the work of Baldwin, Lowry and Thiessen ('51), who observed a progressive increase in the protein efficiency of autoclaved casein-dextrose mixtures as the proportion of water in the mixtures increased.

Despite the great diversity in the experimental methods employed in heating, as reported in the literature, there is a definite conviction that heat may so alter the protein of foods that it suffers a loss in nutritive value. Furthermore, where such losses occur, they have been shown to be largely connected with the reaction of reducing sugars with the protein during the heating process (Patton and Hill, '48; Patton, Hill and Foreman, '48). Assuming that this reaction takes place, the present study demonstrates that the presence of water even in minimum amounts exerts a retarding influence upon the interaction of carbohydrate and protein regularly brought about by heat in the dry state.

In a previous report (Schroeder, Iacobellis and Smith, '53) it was suggested, on the basis of nitrogen balance experiments, that the nutritive value of milk proteins was enhanced by the heat treatment incident to the manufacture of evaporated milk. The present *in vitro* experiments on both dried whole milk and on dried skim milk reconstituted to 3.5% protein show that autoclaving at 15 pounds' pressure for 30 minutes results in an increased rate of proteolysis of the milk proteins in these milk samples by crystalline trypsin and chymotrypsin. The evidence from studies on intact animals (Mader, Schroeder and Smith, '49) that the heat effect upon dry proteins results in decreased digestibility receives support from the present study. The data further show that the presence of milk fat has a minimum effect upon the apparent interaction of proteins and reducing sugars.

SUMMARY

Tests on adult dogs have shown that autoclaving non-fat dry milk solids results in a decrease in the digestibility and nutritive index of the milk proteins. In reconstituted milks, the presence of water markedly inhibits the reaction between carbohydrates and protein.

The results of *in vitro* experiments on the heating of dried skim milk and whole milk powder reconstituted with water show an increased rate of proteolysis of the milk protein by

crystalline enzymes. The fat present exerts a minimum effect.

It may be concluded that the heating of milk proteins in an aqueous medium does not decrease the nutritive value of the protein constituents; on the contrary, heat treatment under these conditions appears to enhance the rate of *in vitro* proteolysis.

LITERATURE CITED

BALDWIN, R. R., J. R. LOWRY AND REINHARDT THIESSEN, JR. 1951 Some effects of processing on the nutritive properties of proteins. Food Res., *16:* 107.

DAVIS, R. M., P. RIZZO AND A. H. SMITH 1949 The effect of heat on the nutritive value of lactalbumin. I. Growth on diets containing heated proteins. J. Nutrition, *37:* 115.

HODSON, A. L. 1952 Nutritive value of milk proteins. I. Stability during sterilization and storage of evaporated milk as determined by the rat repletion method. Food Res., *17:* 168.

LOWRY, J. R., AND R. THIESSEN, JR. 1950 Studies of the nutritive impairment of proteins heated with carbohydrates. II. *In vitro* digestion studies. Arch. Biochem., *25:* 148.

MADER, I. J., L. J. SCHROEDER AND A. H. SMITH 1949 The effect of carbohydrate on the nutritive value of heated lactalbumin. J. Nutrition, *39:* 341.

MELNICK, D., AND B. L. OSER 1949 The influence of heat-processing on the functional and nutritive properties of protein. Food Tech., *III* (2): 57–71.

MURLIN, J. R., M. E. MARSHALL AND C. D. KOCHAKIAN 1941 Digestibility and biological value of whole wheat breads as compared with white bread. J. Nutrition, *22:* 573.

PATTON, A. R., AND E. G. HILL 1948 Inactivation of nutrients by heating with glucose. Science, *107:* 68.

PATTON, A. R., E. G. HILL AND E. M. FOREMAN 1948 Amino acid impairment in casein heated with glucose. Ibid., *107:* 623.

POPE, C. G., AND M. F. STEVENS 1939 The determination of amino nitrogen using a copper method. Biochem. J., *33:* 1070.

SCHROEDER, L. J., M. IACOBELLIS AND A. H. SMITH 1953 Heat processing and the nutritive value of milk and milk products. J. Nutrition, *49:* 549.

SCHROEDER, L. J., R. A. STEWART AND A. H. SMITH 1951 The nutritive effect of heating lactalbumin in the presence of various reducing sugars. Ibid., *45:* 61.

WHITNAH, C. H. 1943 Nutritive value of milk protein. Food Res., *8:* 89.

THE RELATION OF PYRIDINE TOXICITY IN RATS TO DIETARY VITAMIN E[1]

E. L. HOVE

Department of Animal Husbandry and Nutrition,
Alabama Polytechnic Institute, Auburn

TWO FIGURES

(Received for publication March 12, 1953)

The toxicity of pyridine for rats was shown by Baxter ('47, '49) to be decreased by dietary supplements of methionine or cystine. It was interesting to note that the basal diet used for much of his work was deficient in vitamin E. Furthermore, this diet contained cod liver oil and only 10% casein in many cases. These two dietary modifications are known to accentuate the vitamin E requirement of the rat.

On a diet similar to this, it has been shown that the toxicity of carbon tetrachloride for rats was markedly decreased, not only by methionine or cystine, but also by α-tocopherol supplements (Hove and Hardin, '51). Subsequently it was demonstrated that dietary α-tocopherol increased the utilization of methionine in methylating guanidoacetic acid to form creatine by liver slices, *in vitro* (Hove and Hardin, '52). Since there is some indication that detoxification of pyridine may proceed via methylation to the N-methyl pyridinium compound (Baxter and Mason, '47, for discussion), it would be logical to expect dietary α-tocopherol to increase the efficiency of this detoxification mechanism.

[1] Published with the approval of the Director of the Alabama Agricultural Experiment Station. This work was performed under Contract N9 ONR 92100 with the Office of Naval Research. Appreciation is expressed to Merck and Company for supplies of the vitamins used in this work. Aided in part by a grant from the National Vitamin Foundation.

These considerations led to a study of the effect of dietary vitamin E on pyridine toxicity in rats.

EXPERIMENTAL

The basal diet contained 10% extracted casein, 76% sucrose, 4% salt mixture,[2] 9% lard, and 1% cod liver oil. Dry vitamins were ground with a portion of the sucrose to furnish the following levels per gram of diet: thiamine, riboflavin and pyridoxine, 5 μg each; calcium pantothenate, 25 μg; niacin, 40 μg; choline chloride, 2 mg; *i*-inositol, 0.2 mg; and 2-methyl 1-4 naphthoquinone, 2 μg. Some of the rats of each group received this diet to which was added 0.01% dl-α-tocopheryl acetate. In one experiment supplements of 0.125% methylene blue, or 0.5% of yeast nucleic acids, were added to the basal diet.

Male albino weanling rats (Sprague-Dawley), individually caged, were fed the diet ad libitum. After 4 to 6 weeks, pyridine (purified by distillation and passage through silica-gel) was administered as 1% of the diet, or by intraperitoneal or subcutaneous injections, as single doses, at levels of 0.5 or 1.0 ml/kg of body weight. A preliminary determination of the LD_{50} of pyridine injected into rats fed a stock diet led to the selection of these levels.

The livers and brains of some of the rats were analyzed for total lipids, tocopherol (as total fat-soluble reducing substances in the Emmerie-Engel reaction), and spectrometric unsaturated fatty acids (Committee Report on Spectroscopy, '49). The lipid was extracted from the organs as follows: The freshly separated organ was minced and homogenized with three times its weight of water. A volume of absolute ethanol equal to that of the homogenate was added, followed by 100 ml of low-boiling petroleum ether. The resulting mixture was shaken for 10 minutes. Analyses were conducted on aliquots of the separated petroleum ether phase.

The Warburg technique was used to establish the effect of pyridine on the oxidation of unsaturated fats, *in vitro*. A

[2] Salmon, W. D., J. Nutrition, *33:* 155 (1947).

fat emulsion was prepared by homogenizing 20 gm of fat with 3 gm Tween 40 [3] and 80 ml of distilled water in a stainless steel micro Waring Blendor cup. To each Warburg vessel were added 0.66 ml of the emulsion, 1 ml of phosphate buffer, 0.01 M, pH 7.4, and 1.34 ml of water or the neutralized solution of the substance under test. When α-tocopherol was added, it was as a stabile water emulsion, using Tween 40. The vessels were shaken at 37°C. in air and the rate of oxygen consumption recorded. The lard used was free from the stabilizers added commercially and contained 5.89% linoleic acid, 0.46% linolenic acid, 0.23% arachidonic acid and 43.6% oleic acid.

The peroxide number was determined by the method of Lea ('39).

RESULTS

The toxicity of pyridine to normal stock rats

Rats fed a complete stock diet to an average weight of 250 gm were injected subcutaneously with increasing levels of pure pyridine. Six rats were used for each level. The rates of death are noted in figure 1. In these rats pyridine at a level of 0.5 ml/kg body weight caused no deaths. The LD_{50} can be estimated to be about 0.9 ml/kg of body weight.

Modification of pyridine toxicity by dietary vitamin E

The effect of dietary α-tocopheryl acetate, methylene blue and yeast nucleic acid on pyridine toxicity is shown in table 1. The rats were fed the 10% casein diet with the indicated supplements for 6 weeks, at which time 8 rats from each group were injected subcutaneously with 0.5 ml of pyridine per kilogram of body weight, and 6 others with 1.0 ml/kg of body weight.

Methylene blue gave the best protection of the three substances tested. This compound was included since Dam and

[3] Atlas Powder Co., Wilmington, Del.

Granados ('51) have shown it to replace tocopherol in several of its functions. Yeast nucleic acid offered some protection, similar in this respect to its protective action against carbon tetrachloride toxicity (Hove, '48).

The protective effect of α-tocopheryl acetate against pyridine toxicity is shown in 4 other experiments summarized

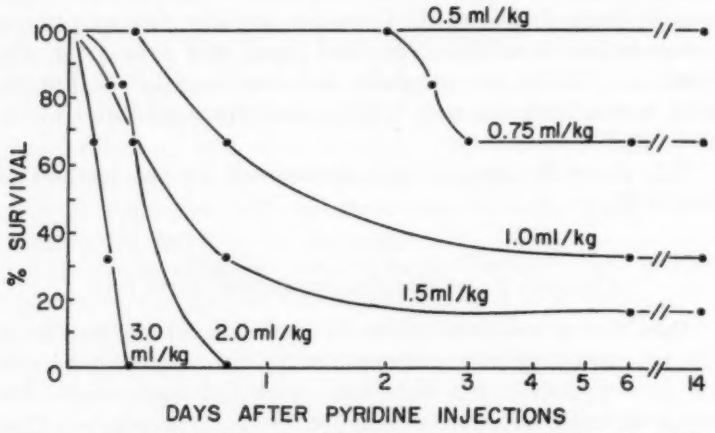

Fig. 1 The graded toxicity of single doses of pyridine injected at the indicated levels, subcutaneously, into adult male rats on a stock diet; 6 rats used per level. The survival (per cent) has been plotted against days after dosing.

TABLE 1

Modification of pyridine toxicity by dietary α-tocopheryl acetate, methylene blue or yeast nucleic acid

ADDITION TO 10% CASEIN BASAL DIET	BODY WEIGHT AT		NUMBER OF RATS ALIVE AT PERIODS AFTER SINGLE PYRIDINE INJECTIONS OF:									
			0.5 ml/kg					1.0 ml/kg				
	Start	6 wk.	0	2	4	6	12	0	6	12	24	48
	gm	*gm*	*days*					*hours*				
None	49.4	85.5	8	6	0	0	0	6	3	0	0	0
α-Tocopheryl acetate (0.01%)	51.8	97.0	8	8	5	2	1	6	5	4	4	3
Methylene blue (0.125%)	50.2	81.0	8	8	5	4	4	6	4	3	3	3
Yeast nucleic acid (0.5%)	48.7	84.5	8	7	3	1	1	6	6	4	3	2

in table 2. In group A pyridine was fed in the low-casein diet at a 1% level, with and without vitamin E, for 4 weeks. Of the 20 weanling rats fed the diet without vitamin E, 15 (or 75%) died at an average time of 11.4 days. The growth of the remainder was only 9.6 gm in the 4-week period. When autopsied, three of the 5 rats still living at 4 weeks showed gross liver damage. The livers were dark mottled red, with some areas almost black. Microscopic examination of stained

TABLE 2

Effect of dietary α-tocopheryl acetate on the toxicity of pyridine administered to rats in various ways

(Rats on the 10% casein basal diet)

GROUP	PYRIDINE ADMINISTRATION	α-TOCOPHERYL ACETATE (0.01% in diet)	NO. OF RATS	INITIAL WEIGHT	4-WK. GAINS	DEATHS		LIVER DAMAGE
						No.	Time	
				gm	*gm*		*days*	*no.*
A	Diet, 1%	—	20	45	9.6	15	11.4	18
		+	20	46	22.1	6	14.0	6
		—	12	180	6.1	3	8.0	3
		+	11	198	18.0	0	..	0
B	Injected 0.5 ml/kg, subcutaneous, single doses	—	11	98	39.0 [1]	8	3.5 [2]	9
		+	7	111	46.2 [1]	0	.. [2]	1
C	Injected 0.5 ml/kg, intraperitoneal, single doses	—	7	66	31.5 [1]	6	2.5 [2]	7
		+	6	65	33.6 [1]	1	2.0 [2]	2

[1] For the 4 weeks prior to injection of pyridine.

[2] Surviving animals killed and autopsied 10 days after pyridine injection.

regions revealed a severe hemorrhagic necrosis. The pathology due to pyridine has been discussed in detail by Baxter ('48). This damage occurred in 18 of the 20 rats (90%) on this treatment. Of the rats fed the diet with added vitamin E, only 6 out of 20 (30%) died, at an average time of 14 days. The growth was depressed to an average of 22.1 gm, or about half of the norm for this diet. In another experiment adult rats were fed the pyridine diet but with less mortality. Of the 12 rats on the diet without vitamin E, three died with

gross liver damage. Of the 11 rats receiving dietary vitamin E, none died.

Data based on the injection of pyridine subcutaneously and intraperitoneally, at the level of 0.5 ml/kg body weight, are presented in table 2 (groups B and C). The protective effect of the dietary tocopherol appeared to be much greater in these experiments than had been previously observed in the

TABLE 3

Composition of liver and brain lipid as effected by pyridine and dietary α-tocopheryl acetate

TISSUE	PYRIDINE INJECTED [1]	α-TOCOPHERYL ACETATE (0.01% in diet)	TOTAL LIPID (Wet basis)	COMPOSITION OF LIPID			
				Oleic acid	Linoleic acid	Arachidonic acid	Total tocopherol
			%	%	%	%	*mg/gm*
Liver	+	0	8.23	11.60	12.74	9.16	0.18
	—	0	4.73	6.46	9.19	9.65	0.31
	+	+	5.16	16.81	10.63	13.91	0.42
	—	+	6.23	29.58	10.00	11.07	1.74
Brain	+	0	4.05	68.3	3.90	10.63	0 (< .05)
	—	0	4.89	59.3	3.54	8.68	0 (< .05)
	+	+	6.70	75.4	5.03	11.03	0.13
	—	+	5.82	59.2	6.04	9.45	0.23

[1] Single doses of 0.5 ml/kg body weight injected subcutaneously into male rats that had been maintained 4 weeks on the basal diet after weaning. Analyses are the average of 4 rats per group 48 hours after the injection.

data given in table 1. The reason for this is not clear. However, a more recently distilled sample of pyridine was used, and this may have a relation to the results.

Summarizing the data in table 2, pyridine treatment of rats without dietary vitamin E caused death in 32 of 50 rats (64%), and liver damage in 37 (74%); similar pyridine treatments of rats receiving vitamin E in their diets caused death in 7 of 44 (16%) and liver damage in 9 (20%).

Table 3 presents data on the unsaturated fatty acids, total lipids and the tocopherol concentration in the livers and brains of rats injected with pyridine. The tissues were obtained 48 hours after injection of 0.5 ml pyridine per kilogram. The

rats had been on their respective diets for 4 weeks from weaning. Pyridine lowered the tocopherol concentration of the tissue fat, but produced no clearly defined alteration in the concentration of unsaturated fatty acids.

The pro-oxidant action of pyridine on lard, in vitro

During the study of the toxicity of pyridine added to the diet at a 1% level, it was noted that such diets became extremely rancid when stored for 10 days in a refrigerator

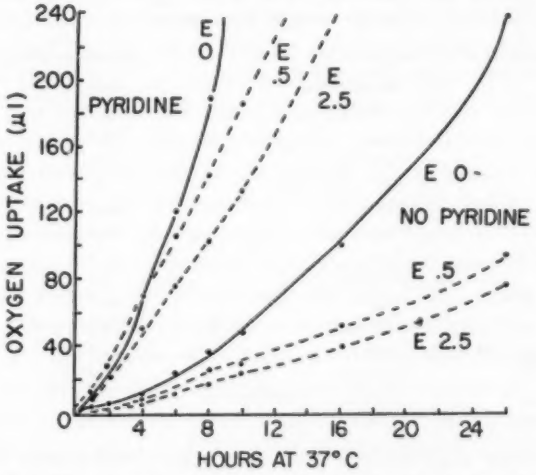

Fig. 2 The effect of pyridine (10 mg) on the oxygen uptake by lard (133 mg) in the Warburg respirometer. Curves *E 0*, *E .5* and *E 2.5* were obtained with dl-alpha-tocopherol added at 0-, 0.5- and 2.5-mg levels in the 3 ml of emulsion, pH. 7.45.

(4°C.). The peroxide number of the fat in the diet had increased to 87 m.e./kg, and the odor of rancidity penetrated even the odor of the pyridine. Identical diets without pyridine had a peroxide number of less than 10 m.e./kg. This observation suggested a pro-oxidant activity of pyridine, with possible implications as to its toxic mechanism.

The pro-oxidant action of pyridine on the lard emulsion is shown in figure 2. Pyridine by itself did not consume oxygen.

The effect of pyridine was not due to its basic nature. Several runs, using buffers or neutralization of the pyridine, established this. The pH values of each of the flasks of the run shown in figure 2 varied only between 7.46 and 7.48. The anti-oxidant activity of added dl-α-tocopherol was relatively ineffective in the presence of pyridine. The lessened activity was possibly not due to destruction of this factor, since pure

TABLE 4

Effect of pyridine and related compounds on the oxygen uptake by various fats in the Warburg respirometer

COMPOSITION OF 3 ML EMULSION [1]		TIME TO ATTAIN O_2 UPTAKE OF:	
Fat	Test compound	50 μl	100 μl
133 mg	*10 mg*	*hr.*	*hr.*
Lard	None	10.6	13.8
Lard	Pyridine	2.8	4.2
Lard	α, α′ dipyridine	3.5	5.3
Lard	Piperidine	10.1	12.9
Lard	Nicotinate	11.5	15.3
Olive oil, U.S.P.	None	24.8	28.5
Olive oil	Pyridine	3.4	4.4
Corn oil	None	> 60	> 60
Corn oil	Pyridine	8.2	10.9
Cod liver oil, U.S.P.	None	2.5	4.3
Cod liver oil	Pyridine	0.23	0.42
Cod liver oil	(Pyridine + methylene blue, 1 mg)	0.90	1.41

[1] Fat emulsion: 20 gm fat, 3 gm Tween 40, 80 ml water, homogenized. Use 0.66 ml emulsion and 2.34 ml of buffer or solution of test compound, adjusted to pH 7.4.

tocopherol dissolved in pyridine retained its full spectral concentration during 48 hours at room temperature.

Further data on the pro-oxidant action of pyridine are shown in table 4. α, α′ Dipyridine was nearly as effective a catalyst as pyridine in the lard system, while piperidine or neutralized nicotinic acid were essentially without action. Pyridine also catalyzed the oxidation of olive, corn, and cod liver oils. It is interesting to note that methylene blue at a 1-mg level considerably reduced the catalytic effect of pyridine on cod liver oil.

DISCUSSION

The toxicity of pyridine to rats appeared to be related to diet in a way closely analogous to the toxicity of carbon tetrachloride. Data in this paper show that on a 10% casein diet, supplements of α-tocopheryl acetate, methylene blue or yeast nucleic acid offered considerable protection to rats treated with pyridine. Other work (Baxter, '49) has shown protection by methionine, cystine or an increased casein level, while Dinning and co-workers ('50) report that vitamin B_{12} permitted improved growth on a pyridine-containing diet. All of these factors have similar action against carbon tetrachloride poisoning (*cf.* Hove and Hardin, '51). The toxicities of the two compounds when expressed as the approximate LD_{50} for stock rats are of about the same order, if the values of 0.9 gm pyridine, and 2 gm carbon tetrachloride per kilogram body weight are expressed on a molar basis.

The mechanism of the toxic action of these compounds is obscure. Pyridine depressed the tocopherol concentration in liver and brain, and Hove and Hardin ('52) reported that carbon tetrachloride markedly accentuated vitamin E deficiency in rats. Daft, Endicott, Ashburn and Sebrell ('43) reported that muscular necrosis produced in rats by succinylsulfathiazole could be prevented by vitamin E, and Bloch ('42) demonstrated similar protection against dystrophy in rabbits due to tri-o-cresyl phosphate poisoning. Since pyridine is methylated in many species, it has been tempting to ascribe its toxicity to a draining of labile methyl needed in the many methylation reactions of the body. Arguments against this hypothesis have been presented by Baxter and Mason ('47).

In the absence of a better hypothesis, the pro-oxidant activity of pyridine in unsaturated fat systems, as demonstrated by the data presented in this paper, may form the basis of a more realistic understanding of the toxic mechanism. Why pyridine should be a pro-oxidant is in itself obscure, and indeed this property may be due to an impurity rather than

to the solvent itself; this also applies to the toxicity of the compound. Against the pro-oxidant theory of toxicity may be cited the lack of any clear alteration in the concentration of unsaturated fatty acids in liver and brain of the poisoned rats. However, the amount of fatty acid peroxides required to disrupt vascular permeability, poison enzyme systems, and kill the animal may be so small that resultant diminution of the unsaturated fatty acids pool cannot be measured by the method employed. Peroxide development in the tissues of vitamin E-deficient animals has been shown by Dam ('49), who postulates a causative relation between the initiation of fat-peroxide formation and damage to capillaries and other symptoms of the deficiency.

SUMMARY

Rats were reared on a 10% casein diet with or without the addition of α-tocopheryl acetate, and given pyridine either in the diet or by injection. Of the rats not receiving vitamin E, pyridine at the levels used caused the death of 64% and produced liver damage in 74%. Of the rats receiving vitamin E, the same pyridine treatment resulted in death in 16% and liver damage in 20%.

Methylene blue and, to a lesser degree, yeast nucleic acid also provided protection against pyridine poisoning. The analogy with dietary substances effective against the toxicity of pyridine, carbon tetrachloride and other compounds is pointed out.

Pyridine was found to catalyze the oxidation of lard and other fats, as measured by oxygen consumption *in vitro*, and to depress the anti-oxidant activity of α-tocopherol. α, α^1-Dipyridine had a similar action, but piperidine and nicotinic acid were inactive.

A toxic mechanism is suggested for pyridine based upon the production of small amounts of fatty acid peroxides in the animal body as a result of the pro-oxidant nature of pyridine.

ACKNOWLEDGMENTS

Appreciation is expressed to Professor D. H. Copeland for his assistance in interpretation of the gross and microscopic pathology of the rats treated with pyridine, and to Mrs. Sarah Sims for assistance in conducting the Warburg studies.

LITERATURE CITED

BAXTER, J. H. 1947 Studies on the mechanism of liver and kidney injury. III. Methionine protects against damage produced in rats by diets containing pyridine. J. Pharm. Exp. Ther., *91:* 345.

———— 1948 Hepatic and renal injury with calcium deposits and cirrhosis produced in rats by pyridine. Am. J. Path., *24:* 503.

———— 1949 Pyridine liver and kidney injury in rats; the influence of diet with particular attention to methionine, cystine, and choline. Bull. Johns Hopkins Hosp., *85:* 138.

BAXTER, J. H., AND M. F. MASON 1947 Studies on the mechanism of liver and kidney injury. IV. A comparison of the effects of pyridine and methyl pyridinium chloride in the rat. J. Pharm. Exp. Ther., *91:* 350.

BLOCH, H. 1942 Der Einfluss von Vitamin-E Mangel auf die Aktivität der Choline-esterase. Helv. et chim. acta, *25:* 793.

COMMITTEE REPORT ON SPECTROSCOPY 1949 J. Am. Oil Chem. Soc., *26:* 399.

DAFT, F. S., K. M. ENDICOTT, L. L. ASHBURN AND W. H. SEBRELL 1943 Vitamin E deficiency in rats given succinylsulfathiazole in purified diets. Proc. Soc. Exp. Biol. Med., *53:* 130.

DAM, H. 1949 Relationship of vitamin E deficiency to tissue peroxides. N. Y. Acad. Sci. Ann., *52:* 195.

DAM, H., AND H. GRANADOS 1951 The influence of certain substances on massive hepatic necrosis and lung hemorrhage in rats fed low-protein, vitamin E deficient diets. Acta Pharm. Toxicol., *7:* 181.

DINNING, J. S., C. K. KEITH, J. T. PARSONS AND P. L. DAY 1950 The influence of PGA and vitamin B_{12} on the metabolism of pyridine-fed rats. J. Nutrition, *42:* 81.

HOVE, E. L. 1948 Interrelation between α-tocopherol and protein metabolism. III. The protective effect of vitamin E and certain nitrogenous substances against CCl_4 poisoning in rats. Arch. Biochem., *17:* 467.

HOVE, E. L., AND J. O. HARDIN 1951 Effect of vitamins E, B_{12} and folacin on CCl_4 toxicity and protein utilization in rats. Proc. Soc. Exp. Biol. Med., *77:* 502.

———— 1952 Creatine synthesis by rat liver, *in vitro*, as influenced by CCl_4 and dietary vitamin E. J. Pharm. Exp. Ther., *106:* 88.

LEA, C. H. 1939 Rancidity in Edible Fats. The Chemical Publishing Co., Inc., New York, N. Y., p. 107.

THE RELATION OF CALCIUM-TO-PHOSPHORUS RATIO TO THE UTILIZATION OF THESE MINERALS BY 18 YOUNG COLLEGE WOMEN [1,2]

MARY BROWN PATTON AND EVA DONELSON WILSON [3]
Department of Home Economics, The Ohio Agricultural Experiment Station, Columbus
AND
JANE M. LEICHSENRING, LOANA M. NORRIS AND CHARLOTTE M. DIENHART [4]
School of Home Economics, University of Minnesota, St. Paul

(Received for publication February 19, 1953)

The ratio of calcium to phosphorus and the amount of vitamin D in the diet have been shown to be factors in the development and cure of rickets in both experimental animals and young children. Based upon observations among several species, Sherman recommends a calcium-to-phosphorus ratio somewhere between one and two during rapid growth and calcification ('46). Macy has stated that insufficient data are available to show the significance of this ratio in food in relation to the retention of calcium during growth ('42). In a discussion of the addition of calcium salts to the diet of pregnant and lactating women, Shohl suggests that the effects of diets with disproportionate amounts of calcium and phosphorus should be tried experimentally at various intake levels ('39).

[1] Journal article 38-52, Ohio Agricultural Experiment Sation.

[2] Paper 2878, Scientific Journal Series, Minnesota Agricultural Experiment Station.

[3] Present address: School of Home Economics, The Pennsylvania State College, State College.

[4] Present address: College of St. Catherine, St. Paul, Minn.

Leichsenring et al. reported an earlier study made in our laboratories which showed that increasing the calcium intake without altering the phosphorus intake resulted in a greater increase in calcium retention than when both calcium and phosphorus were increased ('51). Phosphorus retention was not influenced by increasing the intakes either of calcium or of both calcium and phosphorus. Further study of the significance of the calcium-to-phosphorus ratio in the utilization of these minerals seemed warranted. Eighteen young adult women enrolled as students at the University of Minnesota and at The Ohio State University served as subjects in a study in which 9 different ratios of these minerals were used.

TABLE 1

Experimental treatments

		SUPPLEMENTS TO BASAL DIET IN MG OF PHOSPHORUS PER DAY		
		0	300	600
Supplements to basal diet in mg of calcium per day	0	1	2	3
	600	4	5	6
	1,200	7	8	9

PROCEDURE

An incomplete block, randomized design was set up by the Institute of Statistics of the University of North Carolina in which two levels of calcium and two levels of phosphorus were added in all combinations to a basal diet containing 344 mg calcium and 766 mg phosphorus. The supplements consisted of calcium carbonate, calcium phosphate, sodium phosphate and sodium glycerophosphate and were added in such amounts or combinations as to furnish calcium or phosphorus or both, as indicated in table 1. They were given to the subjects at meal time, approximately one-third of the amount for the day at each meal.

The study extended over a 9-week interval; the first week was termed a "preperiod" and the remaining 8 weeks were divided into 4 parts of two weeks each and were termed "bal-

ance periods." During the second week of each of these two-week periods food, urine and feces collections were made for calcium and phosphorus analysis. The treatments were randomly distributed among the 9 subjects at each school over the preperiod and the balance periods.

The diet was the same as that used in an earlier study and reported by Leichsenring et al. ('51). Chemical analysis of the experimental diet made at the time of the present study showed that it contained an average of 344 mg of calcium and 766 mg of phosphorus. Calculation of other nutrients from food tables showed it was adequate except for riboflavin. To bring the riboflavin intake to the recommended level, each subject received 1 mg of riboflavin daily in tablet form. The diet was further supplemented with 400 I.U. of vitamin D, as recommended by the Food and Nutrition Board of the National Research Council ('48). The foods served varied in kind from day to day but remained the same from week to week. Each ingredient in a mixed food dish was weighed separately for each subject. Foods of low calcium and phosphorus content, such as apples, soda crackers, cookies and sugar candy were allowed ad libitum to supply extra calories when appetites required them.

Composites of food representing a one-day dietary were collected and analyzed for each experimental period. Feces, marked by carmine, were composited for each period for each subject.

The food and feces composites were made into brown digests and later aliquots were wet-ashed according to the method of Stearns ('29). Daily aliquots of urine were made into a composite and preserved by the addition of 5 ml of concentrated HCl daily. Aliquots of the wet-ashed samples of food and feces and the acidified urine samples were analyzed for calcium according to the McCrudden method as modified by Stearns ('29), and for phosphorus by the Fiske and Subbarrow method ('25). Chemical analyses were made of samples of the ad libitum foods and the additional calcium and phosphorus fur-

nished by these foods was added to the calculated intake of each subject. The Ohio subjects drank tap water and the calcium contained in the quantity of water drunk by each girl was determined and added to that in the food and salts. The Minnesota subjects drank distilled water. Both stations used distilled water in food preparation.

The ratios of calcium to phosphorus presented in table 2 are based on the mean values for the foods plus the amounts

TABLE 2

Mean calcium and phosphorus balances of 18 college women on intakes of these minerals in various ratios

TREATMENT NO.	SUPPLEMENTS TO BASAL DIET [1]		CALCIUM-TO-PHOSPHORUS RATIO	MEAN CALCIUM BALANCE	MEAN PHOSPHORUS BALANCE
				mg	*mg*
1	0 Ca	0 P	1: 2.23	— 38 ± 102 [2]	— 16 ± 71
2	0 Ca	300 mg P	1: 3.10	— 46 ± 83	— 13 ± 79
3	0 Ca	600 mg P	1: 3.97	— 16 ± 142	15 ± 63
4	600 mg Ca	0 P	1: 0.81	40 ± 233	— 64 ± 63
5	600 mg Ca	300 mg P	1: 1.13	16 ± 127	24 ± 98
6	600 mg Ca	600 mg P	1: 1.45	52 ± 237	14 ± 146
7	1,200 mg Ca	0 P	1: 0.50	154 ± 211	— 34 ± 79
8	1,200 mg Ca	300 mg P	1: 0.69	155 ± 229	— 62 ± 93
9	1,200 mg Ca	600 mg P	1: 0.88	196 ± 224	86 ± 159

[1] Basal diet furnished an average of 344 mg Ca and 766 mg P from food sources.
[2] Standard deviation.

supplied by salts. The small amounts of calcium and phosphorus supplied by the ad libitum food in individual cases would have very little effect on these ratios.

The young women were within the normal range of weight for height at the beginning of the study and none showed more than the usual fluctuations in weight from week to week. All were reported in normal health by physicians of the Student Health Service at each institution, who examined the young women at the beginning and the end of the study.

RESULTS AND DISCUSSION

Calcium balances

The mean calcium and phosphorus balances on each of the 9 treatments, with standard deviations, are presented in table 2. Each mean is based on balances obtained on 8 subjects, 4 at each location. The treatment numbers and ratios of calcium to phosphorus are also included in this table. Hereafter reference will be made to the treatments by number.

When the calcium retention data were analyzed by the Statistics Laboratory of The Ohio State University by the method of least squares, a treatment effect on calcium retention was evident. To determine which treatments were influencing retention, the differences between all possible combinations of the 9 treatments were calculated and tested for significance.

Balances on treatments 7, 8 and 9 were found to be significantly greater than those on treatments 1, 2, 3 and 5; and those on 4 and 6 were significantly less than that on treatment 9. There were no significant differences in balances due to treatments 7, 8 and 9.

In treatments 4, 7, 8 and 9, the values of the calcium-to-phosphorus ratio were less than 1. The retention of calcium on treatment 4, ratio 1 : 0.81, was significantly less than that on treatment 9, ratio 1 : 0.88 (5% level), but was not significantly less than those on treatments 7 and 8, ratios 1 : 0.50 and 1 : 0.69, respectively. Nor were balances on treatments 7 and 8 different from that on treatment 9.

These results would seem to indicate that calcium retentions are related to the ratio of calcium to phosphorus and are greater when the ratio is less than 1. However, the level of intake is known to be positively correlated to retention, and further analyses were necessary to study the effect on balance of various levels of calcium intake.

Three levels of calcium were used in this study. The first, 344 mg, was supplied by food alone; the second, 944 mg, by food plus 600 mg from mineral supplements; and the third, 1,544 mg, by food plus 1,200 mg from mineral supplements.

At each of these levels of calcium intake, phosphorus was added in the following amounts: 0 mg, 300 mg, and 600 mg. Analyses were made to test the differences in treatment effect on calcium retentions when: (a) calcium is kept constant and phosphorus is increased; and (b) calcium is increased and phosphorus is kept constant. The results of these analyses indicated that, at each level of calcium intake, increasing the phosphorus did not result in a significant increase in calcium retention. However, at each level of phosphorus intake, an increase in calcium resulted in a significant increase in calcium storage.

The absolute amounts of dietary calcium appear to be of greater significance in determining calcium balance than does the ratio of calcium to phosphorus. In discussing the calcium: phosphorus ratio, Sherman ('47) says:

> "The most scientific ground to take on this subject at present would seem to be that somewhat over one gram each of calcium and phosphorus is advantageous in human nutrition; and that with intakes of both these elements at that approximate level we have no occasion for anxiety as to the precise ratio between them."

The regression equation for calcium retention on calcium intake was computed and is the best estimate of the relation of retention to intake available from this study. The regression line crossed the line of equilibrium at an intake of 674 mg.

Two of the treatments used in this study, treatments 7 and 9, were also used among 6 subjects in an earlier study reported by Leichsenring and associates ('51). On treatment 7, approximately 1,500 mg of calcium and 800 mg of phosphorus, the mean calcium retention was 154 mg in the present study and 191 mg in the earlier study. On treatment 9, approximately 1,500 mg of calcium and 1,400 mg of phosphorus, the calcium retention was 196 mg in the present study, whereas the retention in the earlier study was only 33 mg. The addition of phosphorus seemed to have a depressive effect upon calcium retention in the earlier study but this was not apparent in the

present study. The standard deviations of the calcium balances in the present study are evidence of the wide variations among the individuals and these variations may tend to nullify differences due to other causes.

Phosphorus balances

The mean phosphorus balances with standard deviations are presented in table 2. Statistical analysis of these data showed that there was no treatment effect on phosphorus balance among these 18 young women. Further analysis was made to determine whether or not any one treatment was equal in effect to any other. The phosphorus balance on an intake of 1,544 mg calcium and 1,366 mg phosphorus (treatment 9) was significantly greater than that on either 944 mg calcium and 766 mg phosphorus (treatment 4) or 1,544 mg calcium and 1,066 mg phosphorus (treatment 8). The ratio of calcium to phosphorus in treatment 9 was 1 : 0.88; in treatment 4, 1 : 0.81; and in treatment 8, 1 : 0.69.

Phosphorus retentions on treatments 1 (ratio calcium to phosphorus, 1 : 2.23) and 7 (ratio, 1 : 0.50) were significantly less than on treatment 9 (ratio, 1 : 0.88). Retention on treatment 4 (ratio, 1 : 0.81) was significantly less than on treatments 2 (ratio, 1 : 3.10) and 6 (ratio, 1 : 1.45). The wide variations in ratios among treatments that gave significant differences in retention would seem to indicate that factors other than the calcium-to-phosphorus ratio tended to influence retention of phosphorus.

Analyses were made to test the effects on phosphorus balance: (a) when calcium was kept constant and phosphorus was increased; and (b) when phosphorus was held constant and calcium was increased. At the lowest level of calcium intake, 300 mg, increasing phosphorus intake by as much as 600 mg did not increase phosphorus retention. At the two higher levels of calcium intake, 900 and 1,500 mg, increasing phosphorus from 800 to 1,100 mg had no significant effect on phosphorus balance; however, increasing the phosphorus intake to 1,400 mg

resulted in a higher mean phosphorus balance. When phosphorus was kept constant and calcium was increased there was no significant improvement in phosphorus balance.

There are indications that the phosphorus intake level of the basal diet was marginal. At the lowest level of intake, approximately 800 mg, the subjects were losing phosphorus; at the 1,100-mg level, they were losing phosphorus in two cases out of three; and at the highest level, 1,400 mg, they were storing phosphorus.

The regression equation for phosphorus retention on phosphorus intake was computed and is the best estimate of the

TABLE 3

Urinary calcium calculated as percentage of total intake for each of the 9 treatments

	PHOSPHORUS SUPPLEMENT IN MG		
	0	300	600
Calcium supplement in mg	%	%	%
0	34.04	29.71	22.02
600	20.97	19.30	14.72
1,200	15.47	12.90	14.37

relation of retention to intake available from this study. For equilibrium the intake of phosphorus was calculated to be approximately 1,100 mg. As with calcium, the level of intake was a more significant factor in retention of phosphorus among these 18 subjects than was the ratio of calcium to phosphorus.

Urinary excretion of calcium and phosphorus

According to Knapp, the dietary calcium-to-phosphorus ratio influences the urinary excretion of calcium ('47). She reports that urinary calcium, calculated as a percentage of the calcium intake in milligrams per kilogram, showed a consistent increase with increasing calcium-to-phosphorus ratio and constant calcium intake. As shown in table 3, the same pattern is evident from the data reported in this paper. It is most marked

at the 900-mg level of calcium intake (600-mg supplement). At the highest level of calcium intake (1,500 mg), the percentages of urinary calcium were 14.37, 12.90, and 15.47, which show a slight but inconsistent increase from lowest to highest ratio.

The absolute amount of urinary calcium increased with increased intake but the percentage of intake found in the urine decreased with increased intake.

SUMMARY

Eighteen normal healthy young women served as subjects in a calcium-phosphorus balance study designed to determine the effect of varying the ratio of calcium to phosphorus upon the balances of these two minerals.

A basal diet, furnishing 344 mg of calcium and 766 mg of phosphorus, was supplemented by varying amounts of calcium or phosphorus, or both, to give ratios of calcium to phosphorus varying from 1 : 0.50 to 1 : 3.97. The calcium intake levels were 344, 944, and 1,544 mg; the phosphorus levels, 766, 1,066 and 1,366 mg. Sodium glycerophosphate, calcium carbonate, sodium phosphate and calcium phosphate were used as supplements to the basal diet.

The mean calcium balances varied from — 46 to + 196 mg and the mean phosphorus balances from — 64 to + 86 mg.

At each level of calcium intake, an increase in phosphorus intake was without significant effect on calcium retention. However, when phosphorus was kept constant, an increase in calcium intake resulted in a significant increase in calcium balance.

With constant phosphorus intake phosphorus balances were not affected by an increase in calcium intake. An increase in phosphorus intake from the amount in the basal diet, 766 mg, to 1,366 mg resulted in a higher mean phosphorus balance only if the calcium level was as much as 944 mg.

The indications were that the balances of both minerals were more closely related to intakes than to the ratios of calcium to phosphorus.

Urinary calcium, calculated as a percentage of calcium intake in milligrams per kilogram, showed a consistent increase with increasing calcium-to-phosphorus ratio and constant calcium intake.

The absolute amount of urinary calcium increased with increased intake but the percentage of intake found in the urine decreased with increased intake.

LITERATURE CITED

FISKE, C. H., AND Y. SUBBAROW 1925 The colorimetric determination of phosphorus. J. Biol. Chem., *66:* 375.

FOOD AND NUTRITION BOARD 1948 Recommended Dietary Allowances (Revised). National Research Council Reprint and Circular Series, No. 129.

KNAPP, E. L. 1947 Factors influencing the urinary excretion of calcium. I. In normal persons. J. Clin. Invest., *26:* 182.

LEICHSENRING, J. M., L. M. NORRIS, S. A. LAMISON, E. D. WILSON AND M. B. PATTON 1951 The effect of level of intake on calcium and phosphorus metabolism in college women. J. Nutrition, *45:* 407.

MACY, ICIE G. 1942 Nutrition and Chemical Growth in Childhood. Vol. I. Evaluation. Charles C Thomas, Springfield, Ill., p. 164.

SHERMAN, H. C. 1946 Chemistry of Food and Nutrition. The Macmillan Co., New York, N. Y., p. 272.

———— 1947 Calcium and Phosphorus in Food and Nutrition. The Columbia University Press, New York, N. Y., p. 89.

SHOHL, A. T. 1939 Mineral Metabolism. Reinhold Publ. Corp., New York, N. Y., p. 345.

STEARNS, GENEVIEVE 1929 A rapid method for the preparation of fecal digests suitable for use in nitrogen and mineral analysis. J. Lab. Clin. Med., *14:* 954.

THE INFLUENCE OF PROTEIN AND CERTAIN AMINO ACIDS, PARTICULARLY THREONINE, ON THE DEPOSITION OF FAT IN THE LIVER OF THE RAT [1]

A. E. HARPER,[2] W. J. MONSON, D. A. BENTON AND C. A. ELVEHJEM
Department of Biochemistry, University of Wisconsin, Madison

(Received for publication March 9, 1953)

INTRODUCTION

Since Tucker and Eckstein ('37) and Channon, Manifold and Platt ('38) first showed that the deposition of lipids in the livers of rats fed low protein rations deficient in choline was influenced by the methionine and the cystine content of the diet, there have been many attempts to demonstrate a similar action for other amino acids.

Several groups of workers (Channon et al., '38, '40; Best and Ridout, '40; Tucker and Eckstein, '38) have suggested that some proteins are more effective in reducing the liver lipids which accumulate during choline deficiency than would be expected from their methionine and cystine content. Later, however, Tucker, Treadwell and Eckstein ('40) reported that the lipotropic effects of proteins could be accounted for solely by their cystine : methionine balance. The extensive studies

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from Swift and Company, Chicago, Illinois, the Gelatin Research Society of America, Inc., New York, N. Y., and the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

We are indebted to Merck and Co., Rahway, N. J., for crystalline vitamins; to Wilson and Co., Chicago, Ill., for gelatin; to the Abbott Laboratories, Chicago, Ill., for haliver oil; and to Dr. R. A. Collins of the International Minerals and Chemical Corporation, Skokie, Ill., for betaine and glycocyamine.

[2] Permanent address: Department of Biochemistry, University of Alberta, Edmonton, Canada.

stimulated by these observations (Beeston and Platt, '39; Stetton and Grail, '42; Channon, Mills and Platt, '43; Eckstein, '52) failed to produce evidence that specific amino acids other than methionine and cystine influence the deposition of fat in the livers of animals receiving diets deficient in choline.

A few studies of the liver lipids of animals receiving diets containing choline have, however, implicated other amino acids. Singal, Sydenstricker and Littlejohn ('49) reported that the addition of either threonine or *1% choline* reduced the liver fat of rats receiving 9% casein rations. Recently papers from this laboratory (Hawk and Elvehjem, '53; Litwack, Hankes and Elvehjem, '52) have indicated that such factors as the type of carbohydrate, the level of protein and the levels of certain amino acids in the diet may influence the deposition of fat in the livers of rats receiving low casein rations containing both choline and methionine.

The work reported here was undertaken in an effort to extend the latter observations.

EXPERIMENTAL

Male weanling rats of the Sprague-Dawley strain weighing from 40 to 50 gm were used throughout these studies. The animals were divided into similar groups of 6 on the basis of body weight and were maintained in individual cages with raised screen bottoms. They were fed ad libitum and each animal was weighed weekly during the experimental periods, which ranged from two to 5 weeks.

The percentage composition of the basal ration was as follows: sucrose, 81.6; casein, 9.0; corn oil, 5.0; salts IV (Hegsted, Mills, Elvehjem and Hart, '41), 4; methionine, 0.3; choline, 0.13. Vitamins were added to provide, in milligrams per kilogram of ration: thiamine, 5; riboflavin, 5; niacin, 10; calcium pantothenate, 20; pyridoxine, 2.5; folic acid, 0.2; biotin, 0.1; vitamin B_{12}, 0.02; and inositol, 100. Two drops of halibut liver oil, fortified to provide vitamin A, 1,000 I.U., vitamin D, 10 I.U., 2 methyl 1-4 naphthoquinone, 0.04 mg, and alpha-tocopherol,

0.8 mg, were administered weekly. All supplements were included at the expense of sucrose.

At the end of the experimental period each rat was killed by a blow on the head and decapitated and the liver was excised and stored at — 4°C. for the determination of fat. Fat was determined on the dried and ground liver by ether extraction, using the method outlined by Hawk and Elvehjem, ('53).

RESULTS

The results presented in table 1 include the mean weekly gain and the mean per cent liver fat (calculated on both the wet and the dry weight basis) for each group of 6 animals.

Because of the variations observed among the individual experiments, it was not possible to compare all the groups directly, but each experiment included a negative control receiving the 9% casein-sucrose basal ration and a positive control receiving the basal ration supplemented with 6% gelatin.

Experiment 1 was designed to determine the influence of additional protein (2% casein or 6% gelatin) and various combinations of amino acids (table 2) on the level of liver fat. The compositions of the various amino acid mixtures, 4 of which were calculated to simulate portions of the 6% gelatin supplement, are given in table 2. The protein supplements and three of the amino acid mixtures (one, 4, and 5) caused reductions of approximately 50% in the level of liver fat. Amino acid mixtures two and three were without effect.

The object of the subsequent experiments was to determine whether individual amino acids might be effective. In experiment 2 the level of liver fat was appreciably reduced in all groups which received supplements containing either glycine or threonine. The results from experiment 3 further emphasized the effectiveness of these two amino acids in reducing the level of liver fat, threonine being much more effective than glycine. Arginine, proline, tryptophan and diammonium citrate were ineffective; however, when the choline content of the basal ration was increased *three-fold* and the methionine content was raised to 0.5% (groups 11 and 28), some reduction was ob-

TABLE 1

Mean weekly gain and mean per cent liver fat of rats fed low casein rations[1]

EXP.	GROUP	RATION	GAIN/WK.[2]	LIVER FAT	
				Dry wt. basis[2]	Wet wt. basis[2]
			gm	%	%
I	1	9% Casein-sucrose (basal)	15.5 ± 1.7	25.9 ± 2.1	8.4 ± 0.8
	2	11% Casein	27.2 ± 1.3	14.1 ± 1.2	4.1 ± 0.4
	3	Basal + 6% gelatin (acid type)	19.3 ± 2.5	13.1 ± 1.6	3.8 ± 0.5
	4	Basal + amino acid mix 1[3]	18.3 ± 0.8	11.1 ± 0.8	3.3 ± 0.3
	5	Basal + amino acid mix 2[3]	19.4 ± 2.4	24.3 ± 1.1	7.7 ± 0.4
	6	Basal + amino acid mix 3[3]	17.6 ± 1.0	24.0 ± 1.8	7.7 ± 0.8
	7	Basal + amino acid mix 4[3]	14.8 ± 1.3	13.9 ± 0.5	4.1 ± 0.2
	8	Basal + amino acid mix 5[3]	26.2 ± 1.5	11.1 ± 0.8	3.4 ± 0.3
II	9	9% Casein-sucrose (basal)	11.4 ± 1.4	29.1 ± 2.1	9.5 ± 0.8
	10	Basal + 6% gelatin (acid type)	11.2 ± 1.5	15.9 ± 1.5	4.6 ± 0.5
	11	Basal containing 0.4% choline + 0.5% methionine	12.1 ± 1.1	22.2 ± 2.4	6.8 ± 1.0
	12	Basal + 0.56% arginine + 1.5% glycine	9.0 ± 1.6	16.0 ± 2.2	4.5 ± 1.0
	13	Basal + 0.56% arginine + 0.92% proline	12.6 ± 0.7	22.8 ± 2.6	7.1 ± 0.9
	14	Basal + 1.5% glycine + 0.92% proline	11.3 ± 0.8	18.1 ± 1.3	5.0 ± 0.4
	15	Basal + 0.18% threonine + 0.1% tryptophan	22.1 ± 2.2	16.0 ± 1.2	4.8 ± 0.4
	16	Basal + 0.18% threonine + 0.56% arginine + 1.5% glycine	11.7 ± 1.4	12.7 ± 1.0	3.7 ± 0.3
	17	Basal + 0.18% threonine + 0.56% arginine + 0.92% proline	13.3 ± 1.6	14.3 ± 0.8	4.2 ± 0.3
	18	Basal + 0.18% threonine + 1.5% glycine + 0.92% proline	10.6 ± 1.6	11.1 ± 0.9	3.2 ± 0.3
III	19	9% Casein-sucrose (basal)	17.7 ± 1.2	30.1 ± 2.8	10.5 ± 1.2
	20	Basal + 6% gelatin (acid type)	13.3 ± 1.8	10.3 ± 0.2	3.1 ± 0.1
	21	Basal + 6% gelatin + 0.18% threonine + 0.5% glycine + 0.56% arginine	14.5 ± 1.0	10.1 ± 0.5	3.0 ± 0.2
	22	Basal + 0.56% arginine	13.8 ± 0.7	26.2 ± 3.3	8.6 ± 1.1
	23	Basal + 1.5% glycine	16.4 ± 1.8	18.3 ± 0.9	5.5 ± 0.3
	24	Basal + 0.92% proline	15.8 ± 1.3	28.0 ± 2.8	9.2 ± 1.1
	25	Basal + 0.18% threonine	9.7 ± 1.6	12.4 ± 2.0	3.5 ± 0.6
	26	Basal + 0.1% tryptophan	20.9 ± 1.9	31.4 ± 2.8	10.5 ± 1.2
	27	Basal + 2.0% di-ammonium citrate	18.8 ± 1.0	31.5 ± 1.7	10.6 ± 0.8
	28	Basal containing 0.4% choline + 0.5% methionine	14.2 ± 1.6	23.8 ± 1.6	7.6 ± 0.5

	25	Basal + 0.18% threonine			
	26	Basal + 0.1% tryptophan	20.9 ± 1.9	31.4 ± 2.8	10.5 ± 1.2
	27	Basal + 2.0% di-ammonium citrate	18.8 ± 1.0	31.5 ± 1.7	10.6 ± 0.8
	28	Basal containing 0.4% choline + 0.5% methionine	14.2 ± 1.6	33.8 ± 1.6	7.6 ± 0.5
	29	Weanling rats		8.8 ± 1.5	2.5 ± 0.4
	30	9% Casein-sucrose (10 days)	7.0 ± 0.5	23.3 ± 3.3	7.4 ± 1.2
	31	Basal + 6% gelatin (acid type) (10 days)	6.0 ± 1.1	13.7 ± 0.9	4.0 ± 0.3
IV	32	9% Casein-sucrose (basal)	13.3 ± 0.5	34.3 ± 1.8	11.5 ± 0.9
	33	Basal + 6% gelatin (acid type)	8.3 ± 0.6	12.4 ± 1.5	3.5 ± 0.5
	34	Basal + 1.5% serine	8.8 ± 1.0	25.4 ± 1.9	7.6 ± 0.7
	35	Basal + 1.0% glycocyamine [4]	7.7 ± 1.6	25.9 ± 3.9	9.3 ± 1.7
	36	Basal + 1.0% glycocyamine + 0.1% betaine	5.0 ± 0.8	20.9 ± 0.5	7.1 ± 0.3
	37	Basal + 0.56% arginine + 0.92% proline	8.2 ± 0.9	34.3 ± 3.0	12.8 ± 1.5
	38	Basal + 0.56% arginine + 0.92% proline + 0.1% tryptophan	11.2 ± 0.8	34.3 ± 3.2	13.0 ± 1.7
	39	Basal + 0.15% threonine [4]	5.0 ± 0.9	20.5 ± 5.0	7.7 ± 2.3
	40	Basal + 0.30% threonine	5.8 ± 1.2	10.6 ± 0.9	3.1 ± 0.2
	41	Basal + 0.18% threonine + 1.5% glycine	5.8 ± 1.1	8.7 ± 0.9	2.4 ± 0.3
V	42	9% Casein-sucrose + 0.1% tryptophan (basal)	15.3 ± 2.1	34.6 ± 2.9	12.2 ± 1.3
	43	Basal + 6% gelatin (acid type)	25.9 ± 1.5	20.9 ± 1.0	6.3 ± 0.2
	44	Basal + 6% gelatin (alkali type)	25.8 ± 2.9	20.0 ± 0.8	5.9 ± 0.3
	45	Basal + 1.5% glycocyamine [4]	12.7 ± 1.7	25.2 ± 4.2	8.0 ± 1.7
	46	Basal + 1.5% glycocyamine + 0.1% betaine	11.6 ± 0.8	22.2 ± 1.1	6.9 ± 0.4
	47	Basal + 1.0% creatine	17.7 ± 0.7	37.1 ± 3.3	13.3 ± 1.5
	48	Basal + 0.18% threonine	21.7 ± 1.3	16.2 ± 1.3	4.9 ± 0.4
	49	Basal + 0.1% threonine	21.7 ± 0.8	25.3 ± 1.7	8.3 ± 0.6
	50	Basal + 0.1% threonine + 1.5% glycine	20.8 ± 0.6	20.8 ± 1.5	6.4 ± 0.6
	51	Basal + 1.5% glycine	18.2 ± 0.5	25.4 ± 2.5	8.2 ± 0.9
	52	Basal + 2.0% glycine	14.2 ± 0.6	22.5 ± 2.8	7.0 ± 1.2
	53	Basal + 1.5% serine	15.2 ± 0.9	23.7 ± 2.6	7.7 ± 1.0
	54	Basal + 2.0% serine [4]	18.3 ± 1.2	27.9 ± 3.1	9.3 ± 1.2
	55	Basal minus choline	17.7 ± 2.1	42.7 ± 3.6	16.4 ± 1.8
	56	Basal minus choline + 0.18% threonine	23.0 ± 1.6	40.0 ± 3.5	14.7 ± 1.8
	57	Basal 2 weeks + 6% gelatin 2 weeks	25.5 ± 1.4	21.4 ± 1.9	6.6 ± 0.6
	58	Basal + 6% gelatin 2 weeks, basal 2 weeks	21.4 ± 1.7	30.3 ± 1.3	10.2 ± 0.6

[1] Experiment 1 was of 5 weeks' duration; experiments 2 and 3, 4 weeks; and experiments 4 and 5, two weeks.

[2] Each value represents the mean ± the standard error of the mean for a group of 6 animals.

[3] See table 2.

[4] The high mean value for fat and the high standard error for this value are due largely to a single very fatty liver in this group.

served. In neither instance was the liver fat reduced to the values of 12.4% (dry wt.) or 3.5% (wet wt.) which were obtained with the addition of just 0.18% DL-threonine.

The values obtained from the 10-day experiment (groups 30 and 31) indicated that the alteration in liver fat occurred within this time, so the remaining experiments were continued for only two weeks. The growth of the animals in experiment 4

TABLE 2

Amino acid mixtures[1]

	1	2	3	4 NON-ESSENTIAL	5 ESSENTIAL
DL-Alanine			0.60	0.60	
L-Arginine HCl	0.56			0.56	
DL-Aspartic acid			0.37	0.37	
L-Glutamic acid		0.62		0.62	
Glycine	1.50			1.50	
L-Histidine		0.06			0.2
DL-Isoleucine			0.10		
DL-Leucine	0.22				
L-Lysine			0.50		0.4
DL-Phenylalanine	0.24				
L-Proline	0.92			0.92	
DL-Serine		0.20		0.20	
DL-Threonine	0.18				0.2
DL-Tryptophan					0.1
L-Tyrosine			0.012	0.012	
DL-Valine		0.15			0.15

[1] Values represent percentages included in rations.

was somewhat variable (much more so than during the 4- or 5-week experiments) so, since the addition of tryptophan had little effect on the deposition of liver fat, 0.1% of this amino acid was included in all rations in later experiments in order to improve the growth of the animals.

The results of experiment 4 indicate that the inclusion of serine or glycocyamine or glycocyamine plus betaine in the basal ration caused some reduction in liver fat, but these reductions were not as great as that obtained with either 0.3% threonine or 0.18% threonine plus 1.5% glycine.

In experiment 5 the addition of tryptophan to the basal ration resulted in a marked improvement in growth and, since tryptophan was no longer the limiting amino acid, growth responses were obtained when either gelatin or threonine was included. Accompanying the growth response was a general increase, particularly noticeable in the positive control (group 43), in the level of liver fat. All of the individual compounds which had previously been shown to reduce the level of liver fat were effective, although the reductions obtained were not as large as those of the earlier experiments. Threonine again caused the greatest reduction, and again it was observed that glycine increased the effectiveness of a low level of threonine.

The values for groups 55 and 56 demonstrated that the effect of threonine was very slight in the complete absence of choline. The values for groups 57 and 58 indicated that the reduction in liver fat caused by threonine could be reversed if threonine was withdrawn after two weeks, and also showed that the inclusion of threonine for two weeks after rats had been raised on the basal diet for two weeks reduced the accumulation of liver fat. Groups 42 and 43 served as two-week controls for groups 57 and 58.

DISCUSSION

Preliminary histological studies have indicated that by 4 weeks structural alterations occur in the livers of animals receiving the basal ration, and preliminary results from a longer experiment have indicated that the fat accumulation may be transitory. In these two- to 5-week experiments, however, the amount of fat (25 to 35% dry wt.) deposited in the livers of rats receiving the 9% casein-sucrose basal ration was reduced to 10 to 20% (dry wt.) when either the amount of protein or the amount of threonine in the ration was increased. The effect of a low level of threonine was enhanced by the inclusion of glycine in the basal ration. Less marked reductions were obtained when any of the following were included: glycine, serine, glycocyamine, glycocyamine plus betaine or high levels of choline plus methionine. The effect observed with

additional protein confirms the work of Litwack, Hankes and Elvehjem ('52), and the effect observed with threonine confirms the report of Singal, Sydenstricker and Littlejohn ('49).

That the rate of growth has relatively little influence on the fat deposition is demonstrated by comparing groups 9, 10, 15 and 26. The two former groups (basal with and without gelatin) grew at the rate of 11.4 and 11.2 gm/wk., respectively, yet the liver fat of group 9 was 29.1% (dry wt.), while for group 10 the value was 15.9%. Conversely, groups 15 and 26 (basal plus threonine and tryptophan and basal plus tryptophan) grew at the rate of 22.1 and 20.9 gm/wk., respectively, yet the respective values for liver fat were 16.0 and 31.4%. It does appear from the results of experiment 5 that gelatin is somewhat less effective in reducing liver fat than is threonine when the basal ration contains tryptophan. This could be the result of an increased demand for threonine in tissue synthesis, as is indicated by the growth response, when the ration containing gelatin is improved by the inclusion of tryptophan.

In evaluating the results reported it must be kept in mind that the basal ration on which the higher levels of fat were observed contained amounts of choline, methionine, inositol and vitamin B_{12} which are considered to be adequate. It should also be emphasized that Stetton and Grail ('42) have demonstrated that dietary glycocyamine causes increased liver fat deposition during choline deficiency. In these experiments glycocyamine *reduced* the liver fat to about the same extent as did the higher levels of choline and methionine. This, together with the fact that threonine was much more effective in reducing liver fat than choline, indicates that the observations reported here cannot be explained by the generally accepted theories of lipotropic activity.

Dick, Hall, Sydenstricker, McCollum and Bowles ('52) observed pathological changes in the livers of rats fed rations *completely deficient* in threonine, and similar but less marked changes during similar experiments with lysine-deficient animals. They suggested that the histological changes and the slightly increased liver fat deposition were non-specific effects

resulting from an amino acid deficiency and might be due to reduced enzyme formation. In the experiments reported in this paper threonine was not the most limiting amino acid in the diets because no growth response was obtained with threonine unless tryptophan was included in the rations. It is perhaps possible that one amino acid might limit growth but not enzyme activity, while another amino acid which does not limit growth might limit enzyme activity. The reductions in liver fat, admittedly less marked, which were obtained after supplementation with certain non-essential metabolites cannot be readily explained by this hypothesis.

The particular effectiveness of threonine suggests that this amino acid may play a specific role in reducing liver fat deposition under the conditions reported here. The observation of Dick et al. ('52) that lysine deficiency is less effective than threonine deficiency in causing liver changes tends to support this suggestion. If the lesser reductions in liver fat which were observed when the basal ration was supplemented with one of the following: glycine, serine, glycocyamine, glycocyamine plus betaine, excess choline plus methionine, have the same basis as the threonine effect, then a common pathway must be postulated for all of these compounds. It is known that all of them can give rise to glycine and hence also to serine, but again, since threonine is by far the most effective, it is necessary to postulate some other common mechanism or else two distinct mechanisms.

Since all of these compounds can give rise to serine, and since threonine is also an hydroxy amino acid, it is tempting to speculate that the hydroxy amino acids may participate directly in fat utilization. It is well known, of course, that serine is a component of certain phosphatides.

One further point worthy of mention is that gelatin, although an incomplete protein from a nutritional viewpoint, and although it caused no growth response in the absence of tryptophan, was effective in decreasing the accumulation of liver fat observed in rats on these low casein diets. This suggests that criteria other than growth may be important in determining

the supplementary value of such proteins. Gelatins prepared by either the acid process or the alkali process were equally effective.

Although no satisfactory explanation can be provided as yet, there can be little doubt that threonine and the other compounds mentioned influence the deposition of fat in the livers of rats receiving low casein diets containing choline and methionine. It is hoped that further work on this problem may give some insight into the mechanism by which these compounds act.[3]

SUMMARY

The influence of protein (gelatin and additional casein), threonine, glycine, serine, glycocyamine, choline and betaine on the deposition of fat in the livers of rats fed 9% casein-sucrose rations containing adequate choline, methionine, inositol and vitamin B_{12} has been studied. Six per cent gelatin, 2% casein or 0.18% DL-threonine were each effective in reducing the accumulation of liver fat observed in animals receiving the basal ration. The other compounds listed reduced the accumulation of liver fat to a lesser extent under these conditions.

The possibility that enzymes concerned with fat metabolism might have been limiting in animals receiving the basal ration has been discussed, as has also the suggestion that some of these substances may participate more directly in fat metabolism. No satisfactory explanation of the results can be proposed as yet.

LITERATURE CITED

BEESTON, A. W., AND A. P. PLATT 1939 The effect of supplements of tyrosine on the dietary fatty liver. J. Soc. Chem. Ind., *58:* 557.

BEST, C. H., AND J. H. RIDOUT 1940 The lipotropic action of methionine. J. Physiol., *97:* 489.

[3] Shortly after this manuscript was completed, a study of the production of fatty livers in threonine and lysine deficiencies was published by Singal, Hazan, Sydenstricker and Littlejohn in the J. Biol. Chem., *200:* 867–886, 1953. The results of these two studies are in general agreement with regard to the influence of threonine and choline on the deposition of fat in the livers of rats receiving low casein rations.

CHANNON, H. J., M. C. MANIFOLD AND A. P. PLATT 1938 The action of cystine and methionine on liver fat deposition. Biochem. J., *32:* 969.

———— 1940 The action of sulfur-containing amino acids and proteins on liver fat deposition. Ibid., *34:* 866.

CHANNON, H. J., G. T. MILLS AND A. P. PLATT 1943 The action of amino acids and proteins on liver fat deposition. Ibid., *37:* 483.

DICK, F., W. K. HALL, V. P. SYDENSTRICKER, W. MCCOLLUM AND L. L. BOWLES 1952 Accumulation of fat in the liver with deficiencies of threonine and lysine. Arch. Path., *53:* 154.

ECKSTEIN, H. C. 1952 Dietary essential amino acids and the liver lipide content of young white rats. J. Biol. Chem., *195:* 167.

HAWK, E. A., AND C. A. ELVEHJEM 1953 The effects of vitamin B_{12} and B_{12} on growth, kidney hemorrhage and liver fat in rats fed purified diets. J. Nutrition, *49:* 495.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

LITWACK, G., L. V. HANKES AND C. A. ELVEHJEM 1952 The effect of factors other than choline on liver fat deposition. Proc. Soc. Exp. Biol. Med., *81:* 441.

SINGAL, S. A., V. P. SYDENSTRICKER AND J. M. LITTLEJOHN 1949 The lipotropic action of threonine. Fed. Proc., *8:* 251.

STETTON, D., JR., AND F. G. GRAIL 1942 Effect of dietary choline, ethanolamine, serine, cystine, homocysteine and guanidoacetic acid on the liver lipids of rats. J. Biol. Chem., *144:* 175.

TUCKER, H. F., AND H. C. ECKSTEIN 1937 The effect of supplementary methionine and cystine on the production of fatty livers by diet. Ibid., *121:* 479.

———— 1938 The effect of supplementary lysine, methionine and cystine on the production of fatty livers by high fat diets containing cystine. Ibid., *126:* 117.

TUCKER, H. F., C. R. TREADWELL AND H. C. ECKSTEIN 1940 The effect of supplementary cystine and methionine on the production of fatty livers by rats on high fat diets containing casein or edestin. Ibid., *135:* 85.